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INTOXICATION WITH VITAMIN D 895
John Eager Howard and Richard J. Meyer

TRACING THE THYROID HORMONE IN PERIPHERAL TISSUES 911
William T. Salter and MacAllister W. Johnston

THE INCREASED PROTEIN-BOUND BLOOD IODINE IN PATIENTS WITH HYPERTHYROIDISM 934
Roy E. Swenson and George M. Curtis

ASSAY OF THYROTROPIC HORMONE IN HUMAN BLOOD 956
E. De Robertis

THE URETHRAL SMEAR IN THE NORMAL HUMAN MALE 967
Mildred T. Vogel, Thomas H. McGavack and Henry Kammandel

EFFECT OF DL-α-TOCOPHEROL ACETATE ON PROGESTERONE METABOLISM 982
H. de Watteville, R. Borth and M. Gsell

TURNER'S SYNDROME 993
Robert B. Greenblatt and H. E. Nieburgs

ANNOUNCEMENT OF THE ASSOCIATION POSTGRADUATE COURSE IN ENDOCRINOLOGY 1004

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION 1005

ANNOUNCEMENT OF THE AWARDS OF THE ASSOCIATION FOR 1949 . . 1005

NOTES ON THE THIRTIETH ANNUAL MEETING OF THE ASSOCIATION INCLUDING LIST OF OFFICERS, COUNCIL AND COMMITTEE MEMBERS . 1007

ANNOUNCEMENT OF THE VAN METER PRIZE AWARD OF THE AMERICAN GOITER ASSOCIATION FOR 1949 1009

ABSTRACTS OF CURRENT ENDOCRINE LITERATURE 1010

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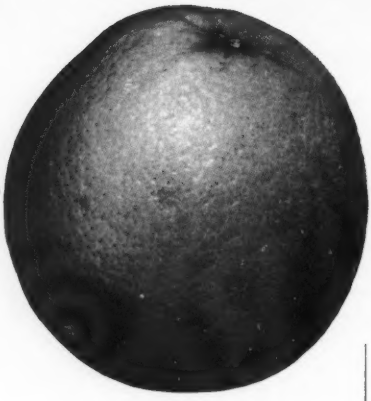

x 16

x 6

} = 1 kilo

a too common denominator

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6 large oranges weigh 1 kilo.

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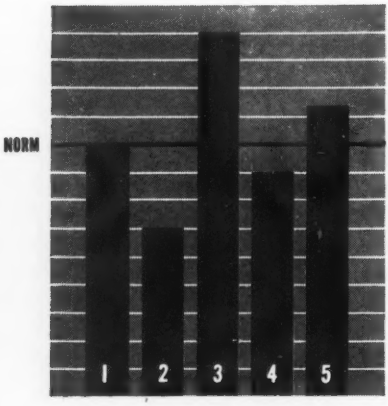

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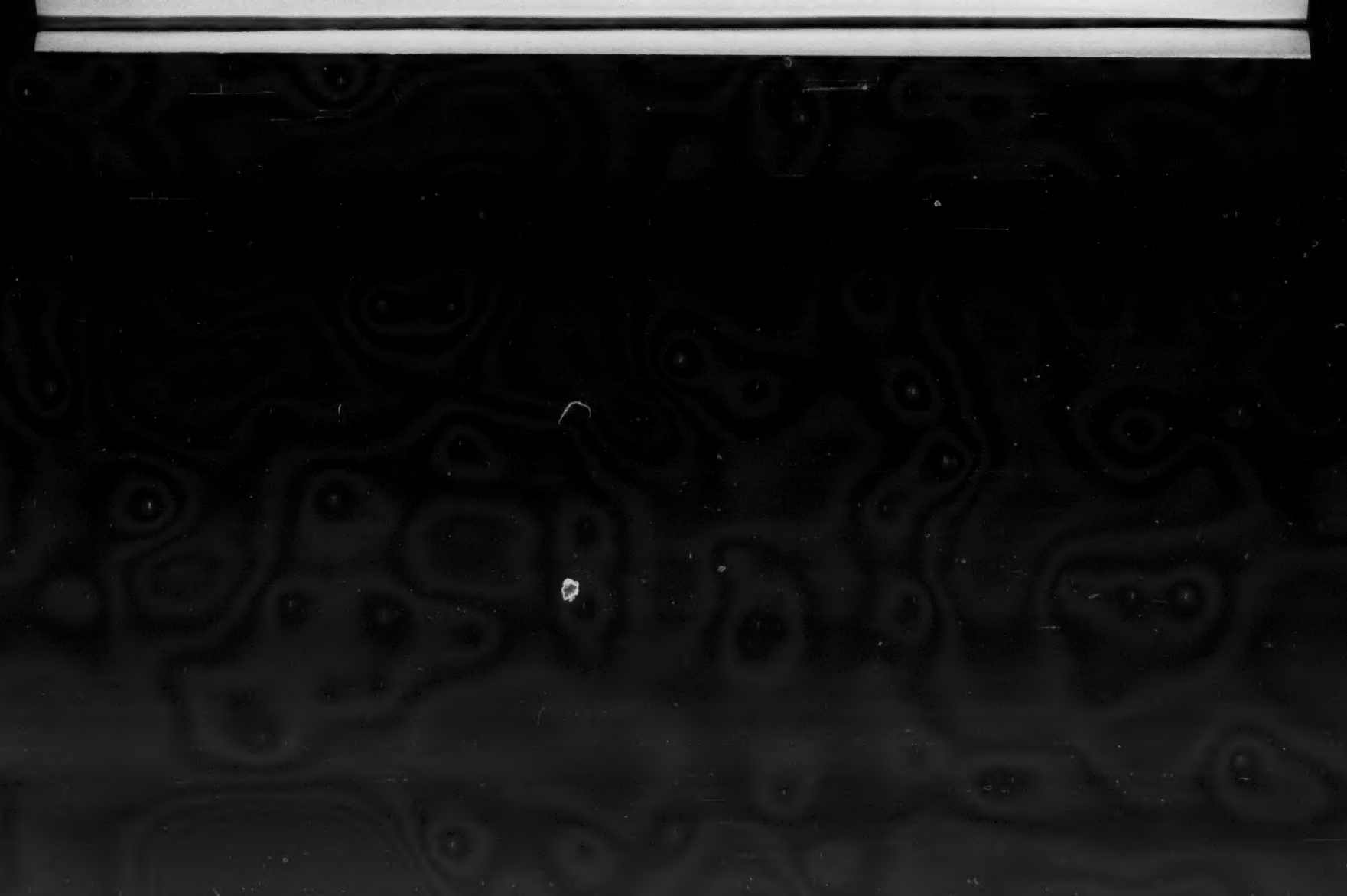

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INTOXICATION WITH VITAMIN D

JOHN EAGER HOWARD, M.D. AND RICHARD J. MEYER, M.D.*
From the Department of Medicine of the Johns Hopkins University and the Medical Clinic of the Johns Hopkins Hospital, Baltimore, Maryland

INTRODUCTION

DESPITE a wide publicity in the medical literature on the dangers associated with the administration of large doses of preparations of vitamin D, (1–12) there are still many physicians who use these drugs without the necessary precautions which should be observed with substances potentially so toxic. During the past three years there have been recognized by us 11 instances of intoxication by such sterols. Ten of these patients had been given the drugs by practicing physicians who were not aware that the alarming symptoms exhibited were related to the therapy which they themselves were prescribing. The eleventh patient was one in whom we ourselves induced hypercalcemia with calciferol (crystalline vitamin D_2) in an extraordinarily short period of time (fourteen days). Analysis of the observations made on these 11 patients forms the subject of this report.

AGES OF PATIENTS, DOSAGE AND DURATION OF THERAPY PRIOR TO SYMPTOMS

The age of the 10 patients who had been given the drug as a therapeutic measure against arthritis varied from 33 to 68 years. Except for the youngest, all were more than 40 years old. Five of the group were males; five were females. Four had received "Ertron," two "Deltalin," two "Davitin," one "Dalsol" and one a combination of "Ertron" and "Darthronal."[1]

Received for publication July 7, 1948.

* Research Fellow, sponsored by Office of Naval Research.

[1] Ertron—capsules (Gelseal), Nutrition Research Laboratories, Chicago, Illinois.
Davitin—tablets (gelatin sealed), International Vitamin Corporation, New York City.

The highest daily dose received by any patient was 600,000 I.U.; the lowest dose was 150,000 I.U. Duration of therapy prior to onset of toxic symptoms was highly variable, ranging from two months to eighteen months. The patient who received the largest daily dose, 600,000 I.U., became ill earliest; but the patient who received the next highest dose, 500,000 I.U., did not manifest symptoms until he had taken the drug for eighteen months.

PRESENTING SYMPTOMS AND CLINICAL FINDINGS

Eight of the 10 patients had severe gastro-intestinal symptoms, namely, anorexia, nausea and vomiting. Weakness, fatigue and lassitude were prominent complaints in all ten. Weight loss was noted in 8, to a lesser degree in the patients who had lesser gastro-intestinal symptoms. Furthermore, in every instance weight loss and fatigue had been noted prior to anorexia and vomiting, and are, therefore, considered to be symptoms of intoxication with vitamin D per se. In none of the patients was vomiting of sufficient severity to produce alkalosis or to require intravenous therapy.

In 7 of the 10 patients polyuria, polydipsia or nocturia were present, having appeared more or less coincidently with the fatigue and malaise. Intense generalized pruritus was a prominent complaint in two patients.

Despite careful search only one of the patients in this group showed any of the more readily detectable evidences of gross vicarious calcification in periarticular, subcutaneous or renal tissues (4, 8, 9, 12, 13). In this patient they were so unusual as to warrant special mention (*see* following case report). Nine of the 10 patients, however, manifested the characteristic eye lesions described by Walsh and Howard (14). These lesions, seen as yet only in patients with hypercalcemia, are visible by slit-lamp examination, and the deposits beneath the conjunctival basement membranes are thought to be composed of calcium salts.

All ten patients, when first seen by us, were suffering from renal insufficiency (Table 1). Concentration of nonprotein nitrogen in the blood was elevated in all (46–106 mg. per 100 cc.). Concentration of calcium in the serum was elevated in every case, ranging from 12.4 to 15.1 mg. per 100 cc. Total protein of the serum was not abnormal in any case, and the A/G ratio was found normal in the 7 on whom this determination was carried

---

Dalsol—multivitamin capsules, U. S. Vitamin Corporation, New York City.

Deltalin—capsules (Gelsealed), Eli Lilly Co., Indianapolis, Indiana.

Darthronal—multivitamin capsules, J. B. Roerig Co., Chicago, Illinois.

All these preparations are alleged to contain 50,000 international units of vitamin D per capsule.

out. Every patient's urine gave a strongly positive Sulkowitch reaction (15). Values of the inorganic phosphorus of the serum varied between 3.0 and 5.2 mg. per 100 cc.; most were above the normal range in this laboratory but not conspicuously so, in view of the retention of nonprotein nitrogen.

The urine contained small amounts of albumin in 7 of the 10; no albuminuria was observed in 3. The urine of only 4 patients contained a few hyaline and granular casts in the sediment; there were rare RBC seen in the sediment of only 2 patients. In this paucity of abnormal urinary constituents, and also in that only 4 of the 10 patients had slight hypertension (the blood pressure reverted to normal within three weeks after stopping the drug) and none had retinopathy, the clinical pattern of the renal disease resembled that described by Longcope and Winkenwerder (16) in chronic pyelonephritis. Anemia was present in 9 of the 10 patients; microcytic hypochromic anemia in 5, normocytic in 4.

Other tests showed conspicuous reduction in renal function except in case 5. Here PSP excretion was 80 per cent in two hours and urine specific gravity, 1.026. The patient did not seem sufficiently dehydrated to account for the elevated nonprotein nitrogen on this basis. She had had symptoms of nausea and vomiting for six months. On the other hand, the patient in case 1 had symptoms of intoxication for only sixteen days; yet PSP excretion was reduced to 28 per cent in two hours and urine could not be concentrated above 1.010.

CLINICAL COURSE AFTER WITHDRAWAL OF THE DRUG

After the condition was recognized, the only therapy prescribed was cessation of drug administration, "forced fluids" (no patient exhibited any tendency toward water or salt retention and an effort was made to have them drink 4000 cc. daily), and a diet low in calcium. By the latter two measures it was hoped to lower the hypercalcemia as rapidly as possible and to reduce the likelihood of renal calculus formation.

Subjective improvement occurred within two to eight weeks after instituting this regimen—nausea, vomiting and lassitude disappeared, even in those who showed but small objective improvement by a fall in serum calcium or betterment of renal function. Objective follow-up data on these patients leave much to be desired, since most were not residents of Baltimore. Serum calcium fell to some extent within a month in all patients except cases 4 and 10. The patients in cases 2 and 9 had normocalcemia[2] within two months; in case 6 within three months; and in case 5 within four months. In general the serum phosphorus tended to fall, sometimes to

[2] Serum protein concentration remained within normal range in all patients.

TABLE 1

Case	Presenting Features	Serum values after omission of medication: initial and follow-up								Initial Urinalysis		
			NPN mg.%	Ca mg.%	P mg.%	CO_3 mEq./L	Cl mEq./L	Total Prot. Gm.%	Alb. Gm.%	Glob. Gm.%	Alb.	RBC	Casts
Case 1. J.O. JHH #386002 W M 56	Weight loss—20 lbs. in 6 mos. Anorexia, weakness and fatigue. Nausea and vomiting—2 wks. No genito-urinary complaints. No anemia—hgb. 13.1 Gm.	Initial	52	13.9	4.0	—	—	6.2	4.2	2.0	2+	0	0
		After 3 wks.	—	11.6	4.0	—	—	—	—	—	2+	0	
Case 2. J.S. JHH #419594 W M 54	Weight loss—40 lbs. in 6 mos. Anorexia, weakness and vague abdominal pain. Nausea and vomiting—3 mos. Nocturia (3×) for 4 mos. Anemia—hgb. 9.0 Gm.; RBC 3.61; Ht. 28 (microcytic hypochromic)	Initial	89	13.6	4.6	29.2	101.6	6.7	4.7	2.0	1+	0	Occ. hyal.& gran.
		After 2 wks.	27	11.4	3.1	27.6	100.0	6.5	—	—	1+	0	Occ. hyal. & gran.
		After 2 mos.	33	10.4	3.0	—	—	7.1	—	—	0	0	0
Case 3. W.C. JHH #421206 W F 65	Weight loss—30 lbs. in 3 mos. Anorexia, weakness and indigestion—2–3 mos. Nausea and vomiting—2 mos. Nocturia 2-3×. Severe backache and joint pains—6 wks. Slight anemia—hgb. 12.0 Gm. RBC 4.4. Ht. 36 (normocytic normochromic)	Initial	73	15.1	5.2	28.7	98.5	6.6	4.6	2.0	1+	0	Occ. hyal. cast
		After 3 wks.	43	12.7	3.6	—	—	7.0	—	—	0	0	
Case 4. A.W. JHH #194939 W M 59	Weight loss—30 lbs. in 1 yr. Weakness and fatigue. Nausea and vomiting (periodic)—4 mos. General pruritis—4 mos. Polydipsia, polyuria and nocturia (4–5x), 4 mos. Anemia—hgb. 8.6 Gm. RBC 3.2 Ht. 23 (microcytic hypochromic)	Initial	62	14.3	4.6	29.5	101.6	7.0	4.3	2.7	1+	0	Occ. gran. cast
		After 1 mo.	62	13.9	4.1	—	—	7.4	—	—	1+	0	0
		After 3 mos.	68	13.3	4.2	—	—	7.4	—	—	2+	0	0
		After 10 mos.	46	12.2	2.5	25.4	108.1	6.9	—	—	1+	0	0
		After 16 mos.	50	10.6	2.6	22.8	—	7.9	—	—	0	0	0
Case 5. F.H. JHH #168084 W F 40	Weight loss—25 lbs. in 6 mos. Weakness and fatigue. Nausea and vomiting—6 mos. No genito-urinary complaints. Anemia—hgb. 10.5 Gm. RBC 4.31. Ht. 34 (microcytic hypochromic)	Initial	46	13.6	3.9	25.4	107.0	7.1	4.5	2.6	0	0	0
		After 4 mos.	40	10.1	2.2	27.6	96.4	7.4	—	—			
Case 6. L.S. JHH #414730 W F 54	Slight weight loss—4 lbs. Marked indigestion, nausea and vomiting. Headaches, weakness and fatigue. Nocturia (4–6×), 1 yr. Anemia—hgb. 8.8 Gm. RBC 3.63. Ht. 27 (microcytic hypochromic)	Initial	57	14.9	3.5	28.7	107.0	7.0	—	—	0	0	Occ. hyal. cast
		After 3 wks.	45	11.0	3.2	—	—	7.0	—	—			
		After 3 mos.	39	10.8	3.2	—	—	7.0	—	—			
		After 6 mos.	39	11.1	3.6	—	—	7.1	—	—			
Case 7. M.D. JHH #446602 W F 68	Slight weight loss—5 lbs. No G.I. symptoms. Fatigue and weakness. General pruritis during vit. D period. Slight polyuria; left kidney removed 18 mos. previously and was polycystic (other kidney said to be normal). Anemia—hgb. 9.2 Gm. RBC 3.33 (normocytic normochromic)	Initial	53	13.6	3.8	—	—	6.8	4.9	1.9	Tr	0	0
		After 2 mos.	43	11.5	3.6	—	—	6.8	—	—	Tr	0	0
Case 8. J.B. Patient of Dr. W. A. Baetjer W M 45	Weight loss—10 lbs.—6 mos. No nausea or vomiting. Fatigue and weakness, marked frequency, polyuria, polydipsia, nocturia—6 mos. Anemia—hgb. 11.1 Gm. RBC 4.2. Ht. 31 (microcytic hypochromic)	Initial	75	14.9	3.6	—	—	6.5	—	—	0	Occ.	0
		After 10 days	56	13.2	3.6	—	—	5.8	—	—			
		After 4 mos.	35	12.9	2.1	—	—	6.9	—	—			
		After 11 mos.	49	10.7	2.3	—	—	6.6	3.9	2.7			
Case 9. W.K. Patient of Dr. C. R. Austrian W M 62	No weight loss. Anorexia, fatigue and weakness. Intractable nausea—2 wks. Nocturia and frequency. Anemia—hgb. 70%. RBC 3.76 (normocytic normochromic)	Initial	106	12.4	3.0	—	—	—	—	—	Tr	1-3	0
		After 3 wks.	37	9.4	4.0	—	—	—	—	—	—	—	—
		After 4 mos.	30	8.9	3.0	—	—	7.6	4.2	3.4	—	—	—
		After 11 mos.	37	10.0	3.0	—	—	7.2	4.3	2.9	0	Occ.	Occ. gran.
Case 10. D.M. Patient of Dr. J. H. Trescher W F 33	No weight loss. Nausea and vomiting—2 yrs. Fatigue and weakness. Sore eyes for 2 yrs. Nocturia—6 mos. Anemia—hgb. 10.4 Gm. RBC 3.6. Ht. 30 (normocytic normochromic)	Initial	53	13.7	5.0	—	—	6.2	4.8	1.4	Tr	0	0
		After 1 mo.	—	14.4	5.6	—	—	7.0	—	—	—	—	—
		After 3 mos.	—	13.5	4.5	—	—	7.0	—	—	Tr	0	0
		After 6 mos.	—	9.2	2.6	—	—	—	—	—	—	—	—

TABLE 1—*continued*

B.P. mm. Hg	Vit. D preparation and dosage	Length of time vit. D taken before symptoms appeared	Duration of symptoms when diagnosis established	Renal function: initial and follow-up	Metastatic calcification	Length of time for symptoms to subside following withdrawal of vit. D
135/85	150,000 units daily for 4 mos. "Ertron"	3½ mos.	16 days	Fishberg: 1.010 PSP:28% Urea clearance: 38% & 20% No follow-up tests.	None found	Improved considerably during hospital stay. No further follow-up.
160/90 in 2 wks. fell to 125/70	300,000 units daily for 6 mos. "Davitin"	4 mos.	2 mos.	Fishberg: 1.012 PSP: 15% After 2 wks.; PSP: 45% After 2 mos.: PSP: 61% Fishberg: 1.014. Urea clearance: 34% & 52%	Cornea & conjunctivae only. Recession of crystals in 1 mo. Band keratitis less but persisting.	Practically complete return to well-being in 2 wks. Nausea persisted for 2 wks. After 2 mos. all symptoms gone; weight gain, return of alertness & strength. Moderate anemia persists.
158/88 in 3 wks. fell to 120/80	200,000 units daily for 18 mos. "Deltalin"	15 mos.	2 mos.	PSP: 15% Fishberg: 1.010 Urea clearance: 16% & 11% After 3 wks.: PSP: 22% Fishberg: 1.012	Cornea & conjunctivae only. No follow-up examination.	Marked symptomatic improvement during her 3-wk. hospitalisation. Vomiting stopped immediately, backache disappeared; weight gain of 3 lbs. No further follow-up obtained.
110/70	500,000 units daily for 18 mos. stopped 4 mos. before admission "Ertron"	18 mos. Calcium deposits noticed in skin at 13 mos.	4 mos.	PSP: 10% Fishberg: 1.018 Urea clearance: 14% & 26% After 3 mos.: PSP: 16% After 10 mos.: PSP: 33% Urea clearance: 40% & 42% After 16 mos.: PSP: 18%	Cornea & conjunctivae, nail beds, lips, skin of hands and face. Recession of calcifications in 16 mos.	Symptoms minimal. Serum calcium normal in 20 mos. Renal insufficiency persists.
122/78	Dosage not accurately determined. Approx. 150,000 units daily for 12 mos. "Ertron" & "Darthronal"	12 mos.	6 mos.	PSP: 80% Fishberg: 1.026 After 4 mos.: PSP: 80%	Conjunctivae only. Recession of conjunctival crystals in 4 mos.	Improvement very definite after first admission. At 4 mos. follow-up there were no complaints.
170/85 in 3 wks. fell to 140/80	400,000 units daily for 12 mos. "Davitin"	10 mos.	6–8 wks.	PSP: 25% Fishberg: 1.014 After 3 wks.: PSP: 28% After 3 mos.: PSP: 55% After 6 mos.: PSP: 67% Fishberg: 1.022	Conjunctivae only. Recession of conjunctival crystals in 6 mos.	Improvement not immediate but by 3 mos. nocturia had lessened; gastro-intestinal symptoms were abated except for slight p.c. discomfort; anemia markedly improved. Exacerbation of arthritis 4 mos. after vit. D stopped.
120/76	600,000 units daily for 2 mos. "Dalsol"	6 wks.	2 wks.	PSP: 5% Fishberg: 1.010 After 2 mos.: PSP: 20% Fishberg: 1.012	Cornea & conjunctivae only. No follow-up examination	General well-being; anemia and pruritis all greatly improved 2 mos. after vit. D was stopped.
120/80	300,000 units daily (irreg.) for 18 mos. "Ertron"	12 mos.	6 mos.	PSP: 9% Fishberg: 1.010 After 2 wks.: PSP: 40% Fishberg: 1.011 After 11 mos.: PSP: 58% Fishberg: 1.018	Cornea & conjunctivae only. No follow-up examination.	In 2 wks. patient had marked return to well-being. Especially notable was return of strength. Arthritis remained quiescent. Anemia persisted for a while but was completely gone at 11 mos.
204/90 in 3 wks. fell to 140/76	300,000 units daily for 5 mos. "Deltalin"	4½ mos.	2 wks.	Fishberg: 1.011 Urea clearance: 21% & 16% After 11 mos.: PSP: 57%	None found.	In 4 days nausea had disappeared. In 1 wk. patient was asymptomatic. Anemia persisted but gradually improved and was gone at 11 mos. There was return of arthralgia and objective joint swelling within 10 days after vit. D stopped.
120/80	200,000 to 400,000 units daily for 4 yrs. "Ertron"	2 yrs. Soreness of eyes noted first	2 yrs.	No initial renal function test. After 3 mos.: PSP: 22% After 7 mos.: PSP: 25% After 9 mos.: PSP: 45%	Cornea & conjunctivae. Conjunctival involvement still present at 5 mos. No further follow-up.	Within 1 mo. dramatic improvement in well-being. Joints bothered patient less. Eyes much better with loss of soreness. Anemia gradually improved in 9 mos.

below normal figures, with restoration of normocalcemia. The patients with the slowest fall in serum calcium were cases 4, 8 and 10; the first mentioned still had hypercalcemia of 12.2 mg. per 100 cc. fourteen months after stopping the vitamin D preparation. At this time serum phosphorus was 2.5 mg. and nonprotein nitrogen was still elevated at 46 mg. per 100 cc. despite improvement in PSP excretion from 13 to 33 per cent in two hours. In case 8 the patient still had, four months after drug withdrawal, hypercalcemia of 12.9 mg. per 100 cc.; serum phosphorus was 2.1 mg.; nonprotein nitrogen had fallen from 75 to 35 mg.; PSP excretion had risen from 24 to only 40 per cent in two hours; and the patient still could not concentrate the urine above a specific gravity of 1.011.

CASE REPORTS

A brief outline of the development of the intoxication and the course of the disease in **case 4** seems worthy of mention since it presents certain unusual features:

J.H.H. #194939. The patient was a farmer, born in 1887, who had always enjoyed exceptionally good health. His only injury had been a blow to the left eye in childhood. In 1938 he began to have progressive arthritic symptoms involving the ankles, knees, shoulders, wrists, elbows, fingers and hips, in that order. He was admitted to the Johns Hopkins Hospital in April, 1941 where the diagnosis of subacute rheumatoid arthritis was made by Dr. C. W. Wainwright. There was present, low grade fever, no anemia, WBC 11,000. Extensive studies revealed: no evidence of renal damage; blood pressure 135/80; and a normal concentration of the following substances in the serum—nonprotein nitrogen, albumin, globulin, uric acid, cholesterol, calcium, phosphorus. He was given a course of 12 injections of gold thiomalate, at weekly intervals, without the appearance of any renal irritation. He was symptomatically improved when discharged in July, 1941, weight and blood pressure unchanged. Thereafter he was followed in the Arthritic Clinic at intervals until July, 1944, always with normal urinary findings and without anemia. His weight was 158 pounds.

The patient was not seen again until December, 1946. Beginning in February, 1945, his family physician had prescribed vitamin D, 500,000 international units daily. There was symptomatic improvement in his joint symptoms coincident with this medication, and it was continued regularly until early in September, 1946—a period of eighteen months. In August, 1946 itching lesions appeared on the right arm and soon became general in distribution. In the latter part of this same month there was noticed a thin line of yellowish-white deposits along the outer borders of his lips and similar larger plaques appeared beneath the finger nails. The latter were somewhat tender and caused him to stop taking the vitamin D preparation. Since stopping the medication he had noticed several small whitish painless areas beneath the skin of the thenar eminences. Nocturia, present previously only when he drank large quantities of fluid before retiring, now was consistently present, four or five times per night. The patient thought this had begun in August. Slight nausea and anorexia had been experienced.

Physical examination revealed a 33 pound weight loss (from 158 to 125 pounds) and a blood pressure of 110/70. His arthritic status seemed unchanged. The color was good. The remainder of the general examination was not revealing. A dermatologic note by

FIG. 1. Lip lesions (Case 4).

Dr. L. W. Ketron: "Patient presents two conditions; over the trunk, extremities and face are split-pea sized, hyperkeratotic nodules, some excoriated, that resemble prurigo nodularis. The lesions of the lip are limited to the vermilion border. Here one sees little whitish implantations which are roundish, irregular or linear in shape and without any definite infiltration (Fig. 1). Some of these have a slightly yellowish tint. Nothing is seen

FIG. 2. The yellowish patches in the finger nail beds (Case 4).

on the mucous membranes of the mouth or tongue. There are also yellowish-white patches in the finger nail beds (Fig. 2). The plate is apparently not affected. There are also some whitish striae at the base of the thumbs involving an area about the size of a 5-cent piece. These are not definitely developed or infiltrated. I do not believe the eruption over the body has anything to do with the nail or lip condition. Am not sure what these latter lesions are but think they are most likely some sort of a degenerative phenomenon, although some of the members of the staff feel they are xanthoma. A biopsy will be taken from a lesion on one of the thumbs." *Biopsy report:* considerable senile

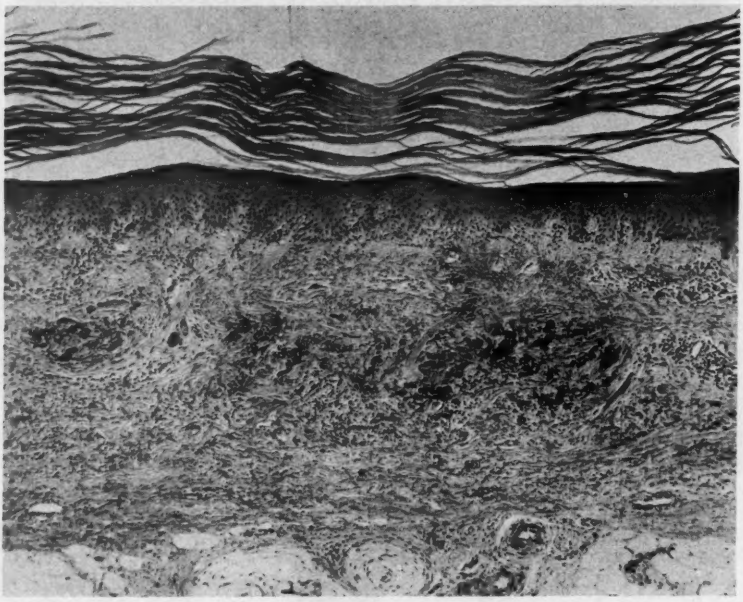

FIG. 3. Biopsy of skin showing senile elastosis with deposits of calcium in the mid-corium (Case 4). ×50 (approx.)

elastosis, with deposits apparently of calcium in a patchy way, in the mid-corium (Fig. 3).

The urine showed a heavy trace of albumin; no abnormal elements in the sediment; and a strongly positive test with Sulkowitch reagent: concentration did not exceed 1.018; PSP excretion was 2 per cent in fifteen minutes and 10 per cent in two hours. Blood non-protein nitrogen was 62, serum calcium 14.3 and phosphorus 4.6 mg. per 100 cc.; serum protein 7.0 Gm. per 100 cc.; and alkaline phosphatase activity, 3.0 Bodansky units. The hemoglobin was 8.6 Gm., RBC 3.2 millions, white blood cell and differential counts were normal, and the sedimentation rate was 16 mm. in one-half hour, corrected. Roentgenograms of the fingers revealed clearly the deposits beneath the nail beds (Fig. 4); but attempts to visualize the deposits in the lips by roentgen ray were unsuccessful. Slit-lamp examination of the eyes by Dr. Frank B. Walsh revealed both the conjunctival crystalline de-

posits and the band keratitis lesions (14). There was a cataract in the left eye, but this was the result of the old injury.

A diagnosis was made of poisoning with vitamin D. He was advised to eat a general diet but to take no milk, cheese or ice cream. Cream and butter were allowed for palatability. He was urged to force the fluid intake to 4 liters daily. He was followed at intervals, and his general symptomatology changed but little. He was able to do moderate farm work, ate and slept well and had no complaints save for mild joint pains. Laboratory data obtained at these visits appear in the following table:

FIG. 4. Roentgenogram of the hands showing the plaques of calcium in the tissues beneath the finger nails (Case 4).

Date	Wt. lbs.	B.P.	Hgb. Gm.	NPN mg. %	Ca mg. %	P mg. %	Alk. phospha-tase (unit)	Prot. Gm. %	Renal function: PSP test 2 hrs.	Urine	
										Albu-min	Sulkowitch test
12/ 9/46	125	110/70	8.6	62	14.3	4.6	3.0	7.0	10%	+	+++
1/20/47	126	110/70	11.6	62	13.9	4.1	3.9	7.4	24%	+	+++
4/ 1/47	125	110/60	12.5	68	13.3	4.2	5.0	7.4	16%	+	+++
6/25/47	125	105/70	13.0	55	13.1	3.7	6.2	7.6	Not done	+	+++
10/28/47	136	120/80	Not done	46	12.2	2.5	Not done	6.9	33%	+	++
4/ 8/48	135	130/80	13.0	50	10.6	2.6	Not done	7.9	18%	0	+

On 4/14/48 the following note was made: "The patient appears just about the same. Skin lesions, though he thinks they are smaller, seem not much changed. It is exceedingly interesting that at last the serum calcium has returned to normal concentration, and coincidently he has had increased arthritic discomfort. He continues to milk his six cows and do gardening."

Dr. Walsh's ophthalmological note: "The cornea is now practically free of any opacity. On the medial aspect of the right cornea is a slight opacity which might pass for a Vogt's white limbus girdle, but even it is very incomplete. The conjunctiva is now free of crystals except for a few small ones that are over the pterygium on the medial aspects of both corneae."

An example of extremely rapid development of intoxication by calciferol is the following:

J.H.H. #366421, a single white woman of 36, had all her life suffered with a complexity of medical difficulties, which in retrospect seemed likely to have all stemmed from unrecognized idiopathic hypoparathyroidism. Because of the main feature of interest here is the rapidity with which she developed hypercalcemia during vitamin D therapy, only the briefest summary will be given. In childhood she suffered with convulsions and attacks of laryngeal stridor. At the age of 26 hysterectomy was performed because of menorrhagia. The following year, because of nervousness, tremor, weight loss and an elevated B.M.R., subtotal thyroidectomy was performed. Because this operation neither relieved the symptoms nor lowered the B.M.R., total thyroidectomy was carried out a few months later, still without benefit. Over the ensuing two years mild hypertension developed and bilateral lumbar sympathectomy was performed. In 1941, at the age of 32, the presence of hypocalcemia was recognized at the Massachusetts General Hospital and the patient was given 1.25 mg. of dihydrotachysterol daily, with partial symptomatic relief. Moderate skeletal rarefaction was discovered and a biopsy of bone disclosed the presence of wide osteoid seams along the bony trabeculae (17). The patient was seen here in 1945. Nutrition, blood pressure, blood morphology and urine were normal. On the routine ward diet, with 2 Gm. of calcium chloride after each meal and 1.25 mg. of dihydrotachysterol daily, the patient was free of tetany and the serum calcium, phosphorus and protein were in normal concentrations. An Ellsworth test (18) was performed; sensitivity to parathyroid extract was disclosed by a sharp rise in urinary excretion of phosphorus.

Calcium chloride and dihydrotachysterol were discontinued and 300,000 I.U. of "Drisdol" were given daily, beginning 11/20/45. The course followed by the concentration of calcium and phosphorus during fasting was as follows:

Date *1945*	Ca mg. %	P mg. %	Prot. Gm. %	
11/20	10.2	3.6	7.4	"Drisdol" begun
11/23	10.1	3.5	7.4	
11/28	10.9	4.5	7.1	
12/4	12.0	6.2	6.9	"Drisdol" discontinued
12/7	15.8	4.3		
12/11	13.8	4.2	7.5	
12/15	12.8	3.6	7.9	
12/17	12.0	3.3	7.4	

On 12/7/45 serum chloride concentration was found to be 90.4 mEq. per liter and serum CO_2, 33.0 mEq. Unfortunately, determination of these substances had not been carried out previously, and we are not in a position to attribute these suggestive evidences of alkalosis to the calciferol intoxication. There had been no vomiting. Nonprotein nitrogen in the blood and urinalysis remained normal. On 12/18/45 Dr. Walsh again examined the patient and found on examination with the slit lamp "in the conjunctivae of the palpebral fissure areas are definite changes. They appear like tiny cysts and deposits of grumous material near the corneal margins."

It was difficult to evaluate the presence or absence of any symptomatic change during the period of hypercalcemia induced by the calciferol. There was a multiplicity of complaints, many of which were felt to be on neurotic and hysterical bases. The patient insisted on discharge from the hospital on 12/19/45. We have the statement of Dr. Milton Little that when the patient arrived home in New England, the conjunctival deposits described above were plainly visible, but re-examination five months later showed that they had entirely disappeared.

DISCUSSION

Though there are many reports in the medical literature attesting the safety of large doses of sterols of this type, it seems clear that serious damage from their use is a frequent occurrence. One drug firm which advertises its product to the medical profession as "safe," attributes its lesser toxicity to the method of manufacture (Whittier process). Though there is reason to believe that this product is more widely used in the treatment of arthritis than other brands of vitamin D, nevertheless 5 of the 10 patients in this series had been intoxicated with this preparation.

The mechanisms by which these drugs may be injurious is an interesting field for speculation, and a rich one for further study. The outstanding symptoms of intoxication with these compounds are fatigue, weight loss, anorexia and vomiting. Impairment of renal function and degenerative lesions with vicarious calcification are the outstanding clinical signs. Since these symptoms and signs are also commonly seen in hyperparathyroidism (19, 20, 21, 22), one is tempted to place the blame on hypercalcemia, which is common to both conditions. The ophthalmic lesions, demonstrated in 10 of the 11 patients of this group, have been found by us only in patients who have had hypercalcemia (14). It seems reasonable to postulate that the hypercalcemia is responsible for similar depositions of lime salts in the other areas of vicarious calcification, and may be the prime cause of the renal insufficiency.

Intoxication with vitamin D, like hyperparathyroidism, seems to induce a situation wherein more than the normal amount of physiologically active calcium is carried in the serum. The older experiments of Jones and Rapoport (23) indicated that irradiated ergosterol greatly enhanced the intestinal absorption of calcium and phosphorus when these two elements were fed independently to the dog. Warkany showed the same in rabbits (24).

Bauer, Marble and Claflin (25) found that in normal persons doses of 30 mg. of irradiated ergosterol lowered fecal calcium and phosphorus, while urinary calcium and phosphorus were increased. Their findings were interpreted to mean that absorption of calcium and phosphorus had been enhanced by the drug. Hypercalcemia would thus seem more likely to result in patients on high calcium diets than on low, especially if the high calcium intake is not accompanied by a high phosphorus intake.

However, Hess and his coworkers found that large doses of irradiated ergosterol produced hypercalcemia in infants and rats even when there were minimal quantities of calcium in the diet (26), and later found this to be true in dogs fed diets absolutely free of calcium (27). Calcium balance was negative in the dogs so treated, and the ash of the bones of rats receiving large doses of irradiated ergosterol was lower than that of control animals (28). Their conclusion was that "the source of the marked increase in serum calcium, induced by excessive amounts of irradiated ergosterol, is the tissues, more particularly the bones which are the great storehouses of calcium in the body." Whereas it might be said that the sterols used by these earlier workers were crude mixtures containing other sterols which might have been responsible for some of their findings, McLean has shown that pure preparations of vitamin D "in calcemic doses" produce resorption of bone and mobilization of bone mineral (29). A large experience with preparations of pure vitamin D over the past 10 years in patients suffering with hypoparathyroidism has convinced us of the correctness of McLean's view (29) that the fundamental precepts mentioned above hold good in patients treated with pure vitamin D_2 (calciferol). A rise in the total and physiologically active components of the serum calcium follows administration of large doses (100,000 to 400,000 units; 2.5 to 10 mg. daily) of calciferol. This rise may be obtained even though minimal quantities of calcium are presented in the diet, but can be greatly enhanced by administration of calcium salts by mouth (30).

The history in the present group of patients did not indicate that any had received high calcium, low phosphorus diets. One patient had received a quart of milk daily on the physician's advice, and another had been given calcium phosphate wafers coincident with the vitamin D therapy. All our patients were given diets very low in calcium immediately on recognition of their condition; yet hypercalcemia was slow to regress. It seems likely that the bones were the major source of the excess calcium in the serum.

A bone biopsy was carried out in only one of our patients. A small wedge-shaped piece of the ilium was removed, in which no abnormality could be detected by Dr. R. H. Follis. This patient had taken 400,000 units of vitamin D daily for a period of twelve months, and conjunctival deposits were readily detected by slit-lamp examination. A sternal biopsy specimen, ob-

tained from a patient previously reported from this clinic, with acute toxicity resulting within twelve days of the administration of very large doses of vitamin D, likewise revealed normal bone (3).

Despite the frequency of digestive complaints, none of the patients in this series took sodium bicarbonate or other alkali. This may be the reason for the absence of more conspicuous vicarious calcification. Hess observed that injection of sodium bicarbonate into dogs poisoned with irradiated ergosterol lowered the serum calcium and brought about reduction of urinary and fecal calcium, but led to increased precipitation of calcium and phosphorus in the tissues of the body (27).

Reversibility of the damage resulting from vitamin D intoxication is of great interest. Others have observed massive deposits of lime salts to disappear after varying periods following withdrawal of the drug (4, 12).

All observers are agreed that the sooner manifestations of vitamin D intoxication are recognized and the drug withdrawn, the more likely will be complete recovery. However, in only two of the patients in our series have we felt recovery to be complete, and in only one of these was there any evidence of renal damage at the time the intoxication was recognized (case 6). In all the others, for the periods in which they have been followed, there have remained some evidences of renal damage, either inability to form highly concentrated urine or reduction in PSP excretion. But if the visible pathologic changes in the eyes are guides to regressive anatomic changes elsewhere, it may be that time will disclose all damage to have been repaired. This possibility seems unlikely, however, especially if the arteries have become calcific.

Using the criterion of continued hypercalcemia as an indicator of persistence of active drug intoxication, the duration of such activity, after the administration of the drug has ceased, seems to be highly variable. In patient No. 4, a period of twenty months elapsed before serum calcium returned to normal concentration. We were concerned lest some unrecognized concomitant process might be present, such as hyperparathyroidism; but there is no evidence that vitamin D poisoning stimulates parathyroid overfunction, and subsequent events have proved our fears to be groundless. Though the calcific deposits in the conjunctivae of this patient were seen to be diminishing gradually at each visit while the serum calcium remained at abnormal levels, it was only at the last visit in April, 1948, when serum calcium was found normal for the first time, that the conjunctival deposits had disappeared entirely. Thus despite his continued renal insufficiency, there may be still hope of further improvement in renal function.

Just as clinical evidence of improvement after drug withdrawal is highly variable as to time, so, too, the dose and duration of administration of the

drugs prior to intoxication seems to rest on factors of individual susceptibility, as yet unknown (1). Many authors have given to patients doses as high or higher than those given to the patients here reported, and over far longer periods of time, without any apparent intoxication (2). Perhaps variations in intestinal conditions may render absorption better in some patients than in others, or there may be more cellular resistance of unknown nature to the action of the drug in some than in others. It has been stated (31) that the effectiveness of certain preparations of vitamin D are greatly enhanced when it is given in milk. None of our patients received it thus.

On the other hand, the great *rapidity* with which the patient with hypoparathyroidism became intoxicated is of interest. We have used preparations of vitamin D almost exclusively for the past 10 years in the treatment of severe cases of hypoparathyroidism. Usually the dose required to maintain normocalcemia in such patients has ranged between 100,000 and 300,000 international units (2.5 to 7.5 mg. calciferol) per day on normal diets without calcium supplements. It was astonishing, therefore, to meet with a rise in serum calcium to 15 mg. per 100 cc. and the rapid deposition of conjunctival deposits in a period of two weeks, using only 300,000 I.U. (3.75 mg.) of the drug daily.

One further curious and unexplained feature in these patients deserves comment. Seven of the 10 arthritic patients insisted that their joint symptoms were improved during the period of vitamin D administration. It is not known how long hypercalcemia had been present before *symptoms* of intoxication appeared, but usually the patients reported that the discomfort in their joints had decreased within two weeks after beginning to take the drug. Many authors have attested to symptomatic relief of arthritis with vitamin D preparations without the production of hypercalcemia (2). It seems unlikely, therefore, that the symptomatic effect on the joints is due to hypercalcemia. After withdrawal of the drug, several patients complained of a sharp increase in arthritic discomfort and this coincided fairly closely with return of the serum calcium to normal. This was especially conspicuous in patient No. 4, whose serum calcium remained elevated for twenty months after drug withdrawal. Coincident with the eventual return to normocalcemia, his arthritis, symptomatically quiescent since beginning the vitamin D therapy, began to give him increasing trouble.

SUMMARY AND CONCLUSIONS

Eleven cases of intoxication by preparations of vitamin D are reported. The symptomatology and pathologic physiology of this condition are discussed. The wide variation in susceptibility of patients to these drugs is emphasized. Ocular manifestations, subconjunctival deposits and lesions

of "band keratitis" may be visible on slit-lamp examination before symptoms of intoxication or signs of renal irritation appear. It is believed that the hypercalcemia is largely responsible for the symptoms and signs of vitamin D poisoning, but the mechanisms by which the hypercalcemia is induced remain obscure. Patients to whom these drugs are administered should be carefully followed by frequent slit-lamp examination, urinary analyses and determinations of the serum calcium.

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TRACING THE THYROID HORMONE IN PERIPHERAL TISSUES*†

WILLIAM T. SALTER, M.D. AND
MACALLISTER W. JOHNSTON, M.D.‡

From the Laboratories of Pharmacology and Toxicology, Yale University School of Medicine, New Haven, Connecticut

THE thyroid is a fascinating gland. Over 120 years ago Hofrichter (1), in speculating on the function of the organ, suggested that it had been provided by nature to adorn the beauty of the neck. Indeed, he called attention to its particular prominence in women and pointed out that men should beware of casting their eyes upon that organ. "Ist es nichts weniger als erwiesen, dass die Schilddrüse zu einer Zeit mehr Blut enthalte, als zu einer anderen—dieses würde der Augenschein lehren, und die Frauen würden schon längst die Mode mit entblösstem Halse zu gehen, verlassen, und die Männer schon längst das Schwellen dieser Drüse als Anzeige der von Seiten der schönen Halfte drohenden Gefahr bemerkt und benutzt haben." This peculiar fascination of the thyroid holds even today for surgeons, physicians and physiologists. They have become so entranced in the mysteries of the gland itself that they have forgotten that, after all, the gland's chief function is to manufacture a hormone—a chemical messenger which it distributes by way of the blood stream to all tissues of the body. What is the nature of this hormone and what function does it serve ultimately?

In recent years, through the experiments of Woolley (2) and of Niemann (3), a certain interest has arisen in the final fate of the hormone and in the possible function it may serve in peripheral tissues. Woolley has sug-

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‡ Arch Hutchison Research Fellow. Permanent address, Toronto General Hospital, Toronto, Canada.

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gested that analogues of thyroxine might be synthesized which would inhibit the peripheral function of the hormone without affecting the gland itself. Niemann also has pondered over possible mechanisms by which thyroxine might control metabolism, and has suggested that a quinoid resonance in the molecule may be responsible for its control over oxidative phenomena. With this increasing interest in the peripheral mechanism of action and in view of the possibility that peripheral thyroid-blocking agents may sometime be available, it seems important to study the ultimate fate of the hormone in peripheral tissues.

Because skeletal muscle constitutes about half of the body mass, it would seem more convenient to attack this particular tissue, but other peripheral tissues (including the blood itself) must be considered as well. The purpose of this report is an attempt to distract attention from the neck and to focus interest upon the organically bound iodine of tissues in the hope that we may learn what thyroxine does there and how it is bound. For instance, it is possible that thyroxine serves as the prosthetic group of a special enzyme system (4) which tentatively we may call "thyrenzyme."

Organic iodine in blood plasma

There is still so much controversial discussion concerning the nature of iodine in erythrocytes that the question will be omitted from the present communication. With respect to the iodine in plasma or serum, however, a good deal of reliable information is now available. First of all, it is clear that under ordinary circumstances the iodide (i.e., inorganic iodine) of the plasma reflects largely the environment or, more particularly, the recent medication or ingesta. In a region relatively free from iodine, the concentration of iodide in the blood and in the body water at large is something like one-half microgram per cent—the barest trace of a "trace substance." If more is found, the increased value is a mere accident or incident, excepting in so far as occasionally it mirrors massive destruction of thyroid tissue, either normal or malignant. It behooves us, therefore, first to consider the organically bound iodine of the plasma—itself a peripheral tissue—not at the moment because of its inherent physiologic interest, but rather because it can offer clues as to how the problem of iodine in other peripheral tissues should be approached.

Among these clues the following suggest themselves:

a) The plasma organic iodine ranges from almost nothing in myxedma to some 20 micrograms per cent in active hyperthyroidism. Indeed, the arbitrary limits usually accepted for normal individuals are about four to eight micrograms per cent. Can corresponding variations in organically bound iodine be demonstrated for peripheral tissues such as skeletal muscle? If so, such a finding would encourage the belief that the concentration

of peripherally bound organic iodine is vital to the behavior and the economy of the organism.

b) It is also important to understand how the thyroxine of the blood is bound chemically, because such knowledge may suggest analogies for the study of tissue iodine. Some investigators (5) have assumed that the blood contains only the free amino-acid thyroxine. Others (6) have assumed that it is incorporated into the plasma protein just as any other amino-acid may be. Because the evidence on this subject is conflicting at present, it seems well to study the behavior of free thyroxine freshly added to plasma.

METHODOLOGY

MICRODETERMINATION OF STABLE IODINE, I^{127}

The method used was that described by Salter and McKay (7), with a few modifications.

In this procedure the organic material is destroyed by dry-ashing *cautiously* in a small muffle furnace. Parenthetically, the dry-ashing requires a certain degree of experience and facility to avoid loss of iodide through superheating. The ash is dissolved in dilute acid, and the *iodide* measured catalytically. The reaction employed was the reduction of ceric ions to cerous ions by arsenious acid, i.e.,

$$2\ Ce^{++++} + H_2O + H_2AsO_3^- = 2\ Ce^{+++} + 2H^+ + H_2AsO_4^-.$$
(yellow) (colorless)

Sandell and Kolthoff (8) showed that the rate of reduction was determined by the concentration of iodide, under *rigidly* prescribed conditions. Moreover Chaney (9) suggested that it might be applied to biologic materials. As shown in Figure 1, the reduction rate is directly proportional to iodide concentration over a considerable range.

Reagents:

Sodium hydroxide (4 normal): 8 Gm. per 50 cc.

Sulphuric acid (1.4 normal): 50 cc. 7 normal H_2SO_4 diluted to a final volume of 250 cc. with distilled water.

Sulphuric acid, "double strength": 80 cc. 7 normal H_2SO_4 diluted to a final volume of 200 cc. with distilled water.

Arsenious acid (0.1 normal): 4.947 Gm. As_2O_3 in 0.01 normal H_2SO_4 per liter.

Ceric ammonium sulfate (0.05 normal): 29.82 Gm. of ceric salt dissolved in 250 cc. of 1.6 normal H_2SO_4.

Potassium nitrate: 10 per cent solution in distilled water.

Potassium nitrate: 1 per cent solution in distilled water.

Acetic acid: (0.2 normal).

Standard solutions:

Stock solution of potassium iodide for known standards: The stock solution contains 261.6 mg. of KI per liter, equivalent to 200 mg. inorganic I^- per liter.

Standard for human serum: For use at 50°C., 1.0 cc. stock/liter—renewed every day. One cc. contains 0.2 microgram of iodide.

Standard for thyroid tissue: For use at 30°C., 1.0 cc. stock/200 cc.—renewed every two days. One cc. contains 1.0 microgram of iodide

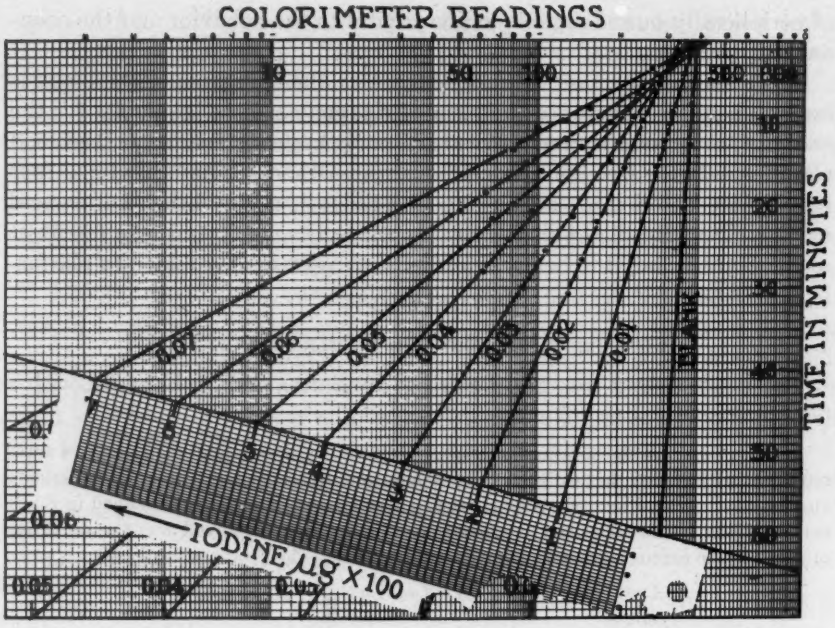

FIG. 1. By plotting the progressive reduction of ceric ions against time upon standard semi-log (three-cycle) graph paper, it is possible to read the iodide content of the analytical sample directly. For this purpose a scale may be prepared by tearing off the base of a companion sheet of graph paper. Obviously the conditions of the determination must be set rigidly.

Standard for whole tissue work: For use at 50°C., 0.5 cc. stock/liter—renewed every day. One cc. contains 0.1 microgram of iodide.

MICRODETERMINATION OF IODINE IN WHOLE SERUM

This method may be used with some variations for specimens of whole serum as well as for those that have been separated into the protein-bound (i.e., "hormonal") iodine and the soluble iodide fractions.

Ashing of serum

In determining iodine in samples of whole serum, 1 cc. of serum is placed in a pyrex test tube 1.3 cm. in internal diameter and 11 cm. long. To it is added 0.5 cc. NaOH solution (80 mg.) and 0.1 cc. 10 per cent KNO_3 solution. This mixture is evaporated to a thin sticky film over a microburner, and thereafter is placed in a small electric muffle furnace (capacity, 4 or 5 test tubes) sold by the Will Corporation, Rochester, New York, and equipped with a pyrometer recording temperatures up to 1100°C. The rheostat is set at "High Heat" and the tubes heated until the pyrometer reaches 520°C. The furnace is then turned off, but the tubes are not removed from the furnace for approximately one-half hour, so that they may cool slowly. When each tube has cooled, 0.5 cc. of the 1 per cent solution of KNO_3 is washed down the sides of the tube, while the tube is rotated.

Next it is dried over the microburner, and again heated in the furnace until it reaches 520°C. It is then allowed to cool in the furnace for one-half hour. This completes the ashing procedure and the sample is ready for the determination of the amount of iodide present.

Much time can be saved if several determinations are performed concurrently. In so doing, the evaporation of the alkaline solutions of protein may conveniently be achieved by fixing the test tubes at an angle a few inches above an electric grill.

Colorimetric procedure

To the contents of the tube is added 3.0 cc. of distilled water followed by 2.0 cc. of 1.4 normal H_2SO_4. The material is next transferred from the oxidation tube to round-bottom colorimeter tubes which have been optically calibrated for a 3.0 cc. volume. Two aliquots are taken from each oxidized sample, i.e., a one-half volume or 2.5 cc. sample, and a one-third volume or 1.7 cc. sample. In the latter case, 0.8 cc. of a blank is added so that the total ionic strength and acidity of the material in the second tube is equal to that of the material in the first tube. This blank contains 0.5 cc. of the NaOH solution, and 2.0 cc. of 1.4 normal H_2SO_4, and is made up to 5.0 cc. with distilled water. Next, into each tube is delivered 0.25 cc. of the arsenious acid solution, and the tubes are mixed by rocking the rack which supports them. The samples are now ready for the colorimeter except for the addition of the ceric ammonium sulfate which is the basis of the reaction. A water bath should be ready to receive the tubes and should be kept at a constant temperature of 50°C. The colorimeter (Klett-Summerson, Corning filter No. 42) is checked at the zero reading of the dial against a blank of distilled water, and a stop watch is at hand.

Time-reaction

At the instant the stop watch is started, exactly 0.125 cc. of ceric ammonium sulfate solution is added to each of the tubes, and they are shaken strenuously and placed in the constant temperature bath. After a lapse of approximately 3 minutes, the initial reading is taken for each tube and the time and reading recorded. Readings are taken at intervals of about 5 to 8 minutes for a 25 to 30 minute period.

Calculation of results

When the observations an the time-reaction are completed, the results are ready for computation. This may be performed 1) algebraically or 2) graphically. In the former instance, the logarithmic difference between the extreme readings for each sample is found on a twenty-inch slide rule and this log-increment is divided by the length of time spanned by the readings. The value so obtained represents the slope or apparent rate of the reaction. This result is translated into micrograms present in the aliquot by using a standard nomogram. In the second instance, the result is read directly from a stereotyped semi-log paper, as shown in Figure 1. With the standard paper shown, a plastic ruler divided into tenths may be used.

Preparation of a standard nomogram

A series of known samples of iodine (i.e., 0.01, 0.02 et cetera, up to 1.0 microgram) are measured accurately and treated exactly as an ashed sample of serum would be treated.[1] After they have been subjected to the colorimetric procedure, the results are calculated

[1] These known samples should not be ashed, because iodide is lost if no organic matter is present.

as above and the points are plotted on ordinary graph paper. That is, the result obtained by dividing the logarithmic difference by the time (i.e., the slope) is plotted opposite the known amount present. After a series of these known points are available, the points that have been plotted will fall in line, and this curve may be used as a standard nomogram. This curve of reference permits very easy interpretation of any further data obtained. It is desirable to have these data calculated statistically so that an exact line may be drawn. Unfortunately such curves tend to be slightly concave upward in the low range of working standards.

Further calculation of the result

The result obtained thus far is only a measure of the iodine present in the aliquot sample, and must be multiplied by two or by three as the case may be, to yield the amount of iodine in the original sample. This value in turn must be adjusted so that it may be translated into micrograms per cent. For example, if only 0.5 cc. of serum is used, the answer must then be divided by that figure (0.5) and then adjusted to 100 cc. in making the final percental calculation.

Separation of "hormonal" (protein-precipitable) iodine from iodide

In separating the protein from blood serum, 1.0 cc. of serum is placed in a combustion tube. The protein may be separated by one of several methods, of which 1) heat-coagulation and 2) precipitation by acetone are most convenient. Of these two, the latter is preferred.

1) *Heat-coagulation.* Approximately 4.0 cc. of distilled water is added to the serum and the contents of the tube are mixed. Approximately 6 drops (i.e., 0.27 cc.) of 0.2 normal acetic acid is added to the tube and the pH of the tube is checked. It should test at pH 6 with a universal indicator paper. The pH may be adjusted by using more acetic acid or by using a dilute NaOH solution. The tube is then placed in a cold water bath, and the bath is brought to the boiling point. The tube is allowed to sit in the bath for about twenty minutes. Then it is removed and allowed to cool to room temperature before it is centrifuged. A flocculant white precipitate will have formed during the heating in the water bath, and this should be stirred away from the walls of the tube before centrifugation. The tube is then centrifuged and the supernatant liquor is poured off into another combustion tube. Next the curd is washed twice with distilled water; and both washes are added to the decanted liquor. Next 0.5 cc. of the standard NaOH solution is added to the curd, which is treated thereafter exactly as a whole blood serum sample is treated. The composite supernatant liquor, which is a large liquid volume (9 to 10 cc.) must be evaporated before it is ready for oxidation. In order to retain the iodide in the solution, the pH value must be alkaline during evaporation. It has been found that prolonged contact with NaOH in the combustion tube produces so much etching of the glass that the colorimetric reaction does not take place smoothly. Therefore 4 or 5 drops of concentrated ammonium hydroxide is used to produce the proper alkalinity in the combustion tube. The tube is placed in a constant temperature oven set for 96° C. This oven is equipped with a vacuum line so that a faster evaporation may be obtained, by drawing air through it continuously. After complete evaporation, 0.5 cc. of the standard NaOH solution is added, and the dissolved coagulum is treated exactly as a whole blood serum sample is treated.

2) *Precipitation with acetone.* The separation of the protein-bound iodine with acetone

is very simple; but the procedure should be performed rapidly in the cold to avoid splitting off the hormonal iodine from its colloidal carrier. To 0.5 or 1 cc. of serum is added 10 cc. of chemically pure acetone during vigorous stirring. Almost immediately there appears a finely divided, discrete coagulum which is readily centrifuged off. The precipitate is washed once with 10 cc. of aqueous acetone and then the iodine in the coagulum is determined. As a test of this procedure one may compare the results obtained in normal and pathologic individuals with those obtained by the routine method of heat-coagulation; and it will be seen that the results agree very well, as shown in Table 1.

TABLE 1. COMPARISON OF SERUM "HORMONAL IODINE" VALUES DETERMINED BY TWO PROCEDURES

Patient	Diagnosis	Serum		
		Inorganic iodine	Organic Iodine	
			Heat-coagulated	Acetone-precipitated
		μg %	μg %	μg %
Cs (Dr. J. Lerman, Boston)	Myxedema	1.7 2.7	2.3 2.9	2.2 2.2
Gi (Dr. R. H. Williams, Boston)	Myxedema	1.7 2.9	2.5 2.9	2.2 2.2
Hl (Dr. J. W. Eweli, New Haven)	Euthyroidism	1.7	4.3 4.6	4.6 5.4
Bh (Dr. McA. Johnston, New Haven)	Euthyroidism	0.9 0.0	4.3 4.1	4.3 4.3
Cn (Dr. K. E. Paschkis, Philadelphia)	Hyperthyroidism	0.5 1.0	16.0 16.6	17.0 17.2

MICRODETERMINATION OF IODINE IN TISSUES

Approximately 300 milligrams of tissue is weighed rapidly, but accurately, in a small glass boat prepared for the purpose. The sample of tissue is transferred to a pyrex test tube and 0.5 cc. of 4 normal aqueous NaOH solution is added, together with 0.1 cc. of 10 per cent KNO_3. Thereafter the treatment is quite similar to the method used for total serum iodine. The resulting suspension is evaporated over a microburner and then ashed in the muffle furnace as described for serum. After cooling, 0.5 cc. of a 1 per cent KNO_3 is added (as described previously) and the liquor is again evaporated before ashing the second time. Occasionally a third ashing is needed.

Colorimetric procedure

After cooling, to the tube (while rotated) are added 1.0 cc. of distilled water and 2.0 cc. of 1.4 normal H_2SO_4. After a few minutes, suitable aliquots of the resultant solution are transferred to colorimetric tubes, i.e., one-half and one-third of the total volume. In the latter case, the final volume is made up to 1.5 cc. with a "blank filler" solution. This "filler" consists of 2.0 cc. of 1.4 normal H_2SO_4 and 0.5 cc. of 4 normal NaOH made up to 3.0 cc. total volume with distilled water. To each colorimeter tube containing the sample is added 0.5 cc. of "double strength" H_2SO_4. Then 0.125 cc. of the ceric ammonium sulfate solution is added, followed by 0.25 cc. of the arsenious acid solution. The stop watch is started as the arsenious acid is added. Then the volume in each tube is brought up to 3.0 cc. precisely, and all tubes are shaken and placed in the water bath at 50°C. Thereafter the method used for whole serum is followed, except that a special nomogram designed *for tissues* is employed in interpreting the rate of reduction.

The yellow color produced in the final reaction mixture increases both with the amount of reagent added and with the acidity. In all determinations the colorimeter reading at zero time should be approximately 350. Therefore, in the more acid media used for tissues, less ceric ammonium sulphate reagent is added than for serum. For analyses of tissues it is convenient to use the same volume of a ceric solution, which is only about 33 per cent as concentrated as that employed with analyses of serum.

Thyroid tissue

Only a few milligrams of thyroid tissue or of minced paste is needed. This small amount is treated as is a sample of blood serum, *except* that the reaction is retarded by operating in a water bath at 30°C. Obviously, a special standard chart designed for 30°C. is needed to interpret the data.

Tissue organic iodine and iodide

In order to separate organic from inorganic iodide, some 300 mg. of tissue (carefully weighed) is ground to a homogeneous suspension after the addition of a convenient amount of distilled water (at least 1.0 cc.). The protein is next separated, either by heat or by acetone precipitation at a pH value close to the isoelectric point.

Measurement of radioactivity

Although radio autographs were made of some of the tissues involved in this study, these results are published elsewhere (10). The measurements of radioactivity reported herewith were obtained with tissue homogenates. Upwards of 250 mg. of fresh tissue, carefully but rapidly weighed in a small glass boat, were ground in a heavy glass conical centrifuge tube fitted with a conical rotary piston. One cc. of distilled water was added. When the suspension appeared finely homogeneous, five volumes of C.P. acetone was added, with continuous stirring, and the heavy albuminous precipitate was spun down. Two successive washings with acetone followed.

The combined clear acetone supernatant liquors contained the inorganic iodine (i.e., iodide). To the mixed liquors was added 1 cc. of 4 normal sodium hydroxide, in a Petri dish 55 mm. in diameter. The acetone was evaporated off to dryness under a drying lamp, thus leaving a thin clear film of tissue extractives.

To the washed acetone precipitate in the heavy centrifuge tube was added 1 cc. of 4 normal sodium hydroxide. The resulting fluid mixture was transferred to a Petri dish of 50 mm. diameter with three 1.0 cc. portions of distilled water. Under the heat of a drying lamp a nearly clear solution was formed and this was evaporated to dryness.

As a general procedure, in measuring radioactivity, each Petri dish is placed under a bell-shaped Geiger-counter chamber (with mica window specified at 30 mg. per sq. cm.), protected laterally with a copper shield. The counts per minute are recorded with a counting rate meter (Massachusetts Institute of Technology) leading to an inkwriter on a moving paper scale, of the Esterline-Angus type. The apparatus is calibrated by means of the pulse-meter described by Mauro and Barry (11) as a "calibrating multivibrator for radio-isotope studies." The background of radioactivity is determined before and after the measurements and these counts per minute are appropriately subtracted from the activity observed. Simultaneous measurements of the original source of isotope are made in each experiment. For this purpose a small fraction of the injected isotope will have been saved as the standard. A portion of this sample is added to each Petri dish containing the inorganic and organic material under analysis. The radioactivity is again measured with the Geiger counter. The difference between the readings before and after the addition of the standard, gives the counts per minute for the standard. These combined readings allow one to calculate the percentage of the injected dose in each sample of tissue.

In the final computation, the data are expressed in terms of 100 grams of tissue. The counts per minute observed are reduced to percentile units of I^{131} administered to the animal or human subject.

BEHAVIOR OF THYROXINE IN SERUM

If the naturally occurring hormone in serum is truly thyroxine, any additional supplement of thyroxine added by an investigator should exhibit the same behavior as the native hormone. Accordingly it is possible to compare two "hyperthyroid" sera. The first is simply the serum of a patient suffering from marked hyperthyroidism. The second is a "myxedematous" serum which has been reenforced with synthetic thyroxine to the same total organic iodine concentration. If the artificial "hyperthyroid" serum fails to behave like the natural specimen, one can conclude that they differ.

When heat-coagulation was applied to the serum of a certain hyperthyroid patient, it was found that the "hormonal iodine" was 16.3 micrograms per cent, whereas the iodide was negligible (0.8 micrograms per cent). By the acetone procedure the value found was 17.1 micrograms per cent. Then to a normal blood serum, containing 4.3 micrograms per cent, was added enough pure thyroxine to raise the concentration to 15.7 micrograms per cent in terms of iodine. Immediately after the addition, the freshly added thyroxine was stirred into the serum and then the reinforced serum precipitated with acetone as previously described. If the free thyroxine so added failed to precipitate with the curd as did the natural iodine, it might be concluded that the bulk of the original "hormonal iodine" in the hyperthyroid blood was not free thyroxine. The results of this manipulation are shown in Table 2 and in Figure 2. In Table 2, also, are given other results of similar experiments performed with sera

from hypothyroid and from normal individuals. These sera were likewise reinforced with an appropriate amount of free thyroxine in solution.

The data so derived indicated a distinct difference between serum from a patient with hyperthyroidism and serum artificially "spiked" with pure

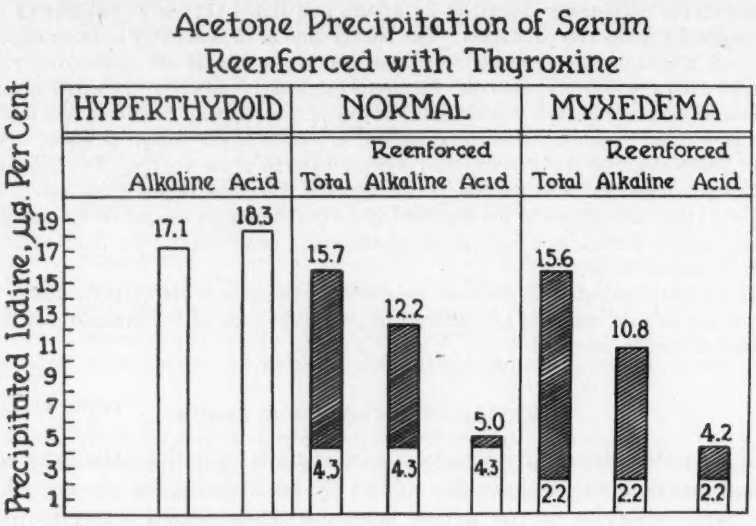

FIG. 2. In serum at pH 8.5 (approximately) a considerable fraction of *added* free thyroxine is precipitated by acetone with the serum protein. At pH 5.5 (approximately), however, very little of the *free* thyroxine is precipitated. Natural serum from a patient with hyperthyroidism behaves in the same way at either pH value.

thyroxine to the same total *organic* iodine concentration. In acid serum (brought to approximately pH 5.5 by adding 0.3 cc. of 0.2 normal acetic acid per cc.), acetone-precipitation yielded different analytical results. It produced substantial removal from solution of the *naturally* excessive hormone, whereas the *artificial excess* of thyroxine remained mostly in solution. The obvious inference is that the excessive hormone in *naturally* hyperthyroid serum is not free thyroxine. On technical grounds, it should be noted from Table 2 that the distinctive behavior of the natural hormone is best demonstrated only after the serum is brought near the isoelectric pH of thyroxine. Otherwise, part of the added thyroxine is precipitated with the serum protein—presumably due to adsorption at the alkaline pH (approximately 8.5) of stored serum which has lost carbon dioxide.

IODINE IN VARIOUS PLASMA PROTEIN FRACTIONS

Protein fractions. As a prelude to investigating the protoplasm of peripheral tissues such as muscle, it would be very helpful to know how the

bound iodine is distributed among the *plasma* protein fractions. If the several protein fractions of plasma were found to have uniform complements of thyroxine, there would be less hope perhaps that the organically bound iodine of peripheral tissues would be consigned to specific carriers. If, on the contrary, it were found that the organically bound iodine traveled preferentially with certain plasma protein fractions, one might also antici-

TABLE 2. "HORMONAL IODINE" OF SERUM BY ACETONE PRECIPITATION METHOD, BEFORE AND AFTER REINFORCEMENT WITH THYROXINE

Patient	Diagnosis	Sample of serum used cc.	Hormonal iodine μg %			
			Heat coagulation	Natural plus thyroxine	Acetone precipitation	
					Before reinforcement	After: reinforcement
					Alkaline	Acid
Cn (Dr. K. E. Paschkis, Philadelphia)	Hyperthyroidism	0.5	16.3	Natural 17.1 Thyroxine + 0.0 Expected 17.1	17.1	18.3
Bh (Dr. McA. Johnston, New Haven)	Euthyroidism	0.5	4.3	Natural 4.3 Thyroxine +11.4 Expected 15.7	12.2	5.0
Cs (Dr. J. Lerman, Boston)	Myxedema	0.5	2.6	Natural 2.2 Thyroxine +13.4 Expected 15.6	10.8	4.2
Gi (Dr. R. H. Williams, Boston)	Myxedema	1.0	2.7	Natural 2.2 Thyroxine + 5.6 Expected 7.8	4.3	
Hl (Dr. J. W. Ewell, New Haven)	Euthyroidism	1.0	4.5	Natural 5.0 Thyroxine + 2.8 Expected 7.8	6.6	

pate the same unequal distribution among the proteins of peripheral tissues.

When the proteins of human plasma are separated either by ammonium sulphate or cold alcohol it is found that the iodine is not uniformly distributed among the various protein fractions. Early in 1938 one of the authors (12, 13) studied horse serum and human serum and showed that after routine precipitations with ammonium sulphate such as once were used to distinguish the so-called albumin from the so-called globulin fractions, the iodine resided preferentially with the albumin fraction. Later through the courtesy of Professor Edwin J. Cohn, studies were made on

carefully prepared fractions of human plasma obtained from the Red Cross (14) and again unequal distribution was found. As far as the total organic iodine is concerned, the larger portion separates with the albumin fractions. However, if one is interested only in the highest concentration, it turns out that the alpha-beta globulin fractions contain the most iodine per gram. Nevertheless this alpha-beta globulin accounts for only a minor part of the total iodine which precipitates with protein. Some of the results obtained with plasma protein fractions purified by Cohn's group are given in Table 3. When compared with the original data obtained with ammonium sulphate and horse serum many years ago their physiologic import appears to be the same. The absolute magnitude of these results is of little significance at the moment. The point which they are intended to illustrate is that the organically bound iodine is not uniformly distributed among all the plasma proteins. With this in mind it will be interesting to see what happens to the organically bound iodine which is found in peripheral tissues.

TABLE 3. UNEQUAL DISTRIBUTION OF BOUND IODINE IN PLASMA PROTEIN FRACTIONS

Classical fraction (approximate)	Cohn's designation, fraction	Per 100 ml. plasma		Iodine concentration (micrograms per 100 grams protein)
		Protein (grams)	Iodine (micrograms)	
Whole plasma		8.4	4.9	82
Fibrinogen	I	0.5	0.2	32
Euglobulin	II & III	2.0	1.2	62
α-β globulin	IV-1	0.5	0.9	190
	IV-4	0.5	0.7	150
Albumin	V	3.2	1.5	48
Total		6.7	4.5	

Because the plasma iodine appeared to hold more closely with the smaller protein molecules, it seemed likely that the protein escaping through the capillaries into the tissue spaces and eventually to the lymph would be relatively more rich in iodine than the mixed plasma proteins themselves. This was actually found to be the case in dog's lymph (15) studied with the help of Dr. Cecil K. Drinker. In fact, per gram of lymph protein there was about twice as much organically bound iodine as in the case of plasma. These findings harmonized well with the observations of Riggs and his collaborators (6), whose studies with the ultracentrifuge showed that the smaller molecules were accompanied by a higher concentration of iodine.

Critique of blood iodine. Considering at this time only the blood plasma,

the following facts are available to guide the investigator in pursuing work with other tissues:

a) The concentration of the plasma protein-bound iodine varies with the thyroid status of the individual.

b) The "hormonal iodine" in plasma does not behave like free thyroxine, although the type of linkage binding the prosthetic group to the carrier remains unspecified.

c) The protein-bound iodine is unevenly distributed among the protein fractions of the plasma.

d) The smaller molecules of protein are richer in iodine; and, as they escape from the plasma into the tissue spaces and thence to the lymph, the proportion of iodine to protein increases.

IODINE IN THE PERIPHERY

Lymph. Intermediate between the blood capillaries and the tissue cells is the interstitial fluid and the lymph which it ultimately becomes. For practical purposes one must be content to study lymph. As already stated, the protein of lymph is richer in iodine than the mixed serum proteins. The organic iodine of cerebrospinal fluid (a special case!) and of lymph from various sources is roughly predictable from the protein content thereof. Thus cerebrospinal fluid contains almost no organic iodine (16), whereas lymph from the thoracic duct contains an amount approaching that of plasma, even in the fasting state.

Iodine in mammalian tissues. It is clear from the work of Wallace and Brodie (17) and others that the inorganic or ionized iodine per gram of water is the same in all tissues including the plasma. In the literature of the last two decades, however, analyses of tissues have been published (18, 19, 20) which indicate that the organic iodine in peripheral tissues is distinctly higher than that of the plasma. Unfortunately, most of these results were obtained by oxidation procedures, followed by distillation; and this type of methodology is likely to be complicated by certain impurities which result from the oxidation of fat. In particular, analyses made of brain tissue, including the pituitary gland, have tended to run high. As shown in Table 4, when various fresh peripheral tissues from rats, cats and human subjects are analyzed, the results are not unlike those obtained in the plasma. Furthermore, when rats were studied in various stages of thyroid activity, as shown in Table 4, the hypothyroid rats showed low values for peripheral-tissue iodine (e.g., muscle) whereas animals injected with thyroxine yielded values much higher than normal. The analytical technique used was the dry-ashing procedure of Salter and McKay (7) slightly modified by the addition of double-strength sulfuric acid in the final colorimetric estimation, as already mentioned.

TABLE 4. IODINE (μg.) IN PERIPHERAL TISSUES

	Organ									
	Muscle							*Liver*	*Kidney*	*Heart*	*Serum*
		Protein Fractions									
	Fresh Tissue	A		B		C		*Total Iodine*			
		I/100 Gm.	Total I	I/100 Gm.	Total I	I/100 Gm.	Total I				
Normal rats (twenty)	6.2 ± 0.9							5.2 ± 1.2	5.2 ± 0.7	6.2 ± 0.9	3.7 ± 0.7
Treated with thiouracil											
1.00 Gm. daily for 10 days	5.0										
1.00 Gm. daily for 28 days	4.9										
0.25 Gm. daily for 90 days	3.2										
0.25 Gm. daily for 105 days	1.8										
Treated with thyroxine											
0.25 mg. daily for 5 days	8.2										
0.25 mg. daily for 11 days	20.0										
0.50 mg. daily for 10 days	21.0										
Normal cats (six)	25.0 ± 6.2							28.0 ± 11.6	27.0 ± 10.4	14.4 ± 28.0	3.3 ± 0.1
Partition by ammonium sulfate		22.2	4.2	73.0	1.0	5.1	0.1				
Per cent partition			66		16		2				
Partition by acetone		98.7	9.7	252.0	1.0	—	—				
Per cent partition			85		8						
Human subjects (two)	5.3 ± 1.0										
Partition by acetone		14.8	0.27	19.5	0.17	64	2.13	5.5	6.4	—	6.8
Per cent partition			11		7		82	2.6	3.0	—	2.8

Fractionation of muscle proteins

When various muscle protein fractions are studied, it is found that the iodine bound to them is not uniformly distributed. As yet the fractionation of muscle proteins is still open to considerable discussion; but a recent monograph by Szent-Györgyi (21) indicates that acetone may be used to separate actin, myosin and other proteins or protein fractions. Experiments made in the present investigation with ammonium sulphate yielded a great deal of insoluble material and therefore this approach was abandoned for the use of acetone. As shown in Table 4, successive fractionation with increasing concentrations of acetone yielded material in three fractions designated as A, B, and C. The "A" fraction was soluble in water at pH 7, the "B" fraction was soluble in water at pH 9, and the "C" fraction was insoluble in 2.0 normal ammonium hydroxide. The distribution of iodine in this protein material indicated that "B" was the highest repository in terms of protein concentration, although not in terms of total amount. Further studies on the fractionation of muscle material are in progress both in cat and in human tissue.

TABLE 5. ACETONE PRECIPITATION OF TISSUE ORGANIC IODINE REINFORCED WITH FREE THYROXINE

Acetone precipitate per 100 grams protein				
	Cat		Human	
	Fraction A	Fraction B	Fraction A	Fraction B*
	μg %	μg %	μg %	μg %
Analyzed				
1) protein precipitate alone	12.7	14.6	14.8	19.5
2) thyroxine added	57.8	93.2	42.4	81.9
3) protein plus free thyroxine	69.0	114.1	57.2	101.7
4) acetone precipitate of *3*	14.4	17.1	16.0	35.0
Calculated				
free thyroxine precipitated by acetone	1.7 (or 3%)	2.5 (or 3%)	1.9 (or 3%)	16.5* (or 19%)

* This fraction was somewhat alkaline (pH 8) and, therefore, could be expected to adsorb some thyroxine.

Free versus bound thyroxine

Obviously the question arises whether the so-called thyroxine in peripheral tissues (e.g., muscle) exists as such or whether it is incorporated into a colloidal system, possibly a peripheral enzyme system. One is reminded of

the fate of thiamine (vitamin B_1), which becomes co-carboxylase in tissue. Accordingly, experiments were designed to test whether the iodine in the muscle protein behaves like free thyroxine. Two typical experiments are shown in Table 5, one dealing with protein fractions from cat muscle and the other with protein fractions from human muscle. In both instances the plan of the experimental approach was the same. First, the iodine bound to the protein was determined and then a known amount of additional free thyroxine was added in a duplicate experiment and treated in exactly the same way with acetone. As shown in Table 5, although each protein fraction (as precipitated by the acetone) contained a complement of natural iodine, the free thyroxine added failed to appear with the precipitate, but remained in the supernatant fluid. These experiments indicate that the organically bound iodine found naturally in muscle is precipitated under conditions which do *not* precipitate free thyroxine. The immediate physiologic inference is that the form of thyroid hormone which resides in peripheral tissues does not behave like free thyroxine. For this colloidally bound tissue iodine the name "thyrenzyme" is suggested.

There has been a school of thought which suggested that free thyroxine is concentrated only in cell membranes and does not penetrate into the protoplasm or cell sap. The fact that these protein fractions (separated by repeated isoelectric precipitation and by "salting out" with acetone or ammonium sulphate) *do* contain bound iodine suggests strongly that the iodine-containing material is combined with at least one cellular constituent. Of course, such chemical data cannot localize the precise site (within or on the cell) where the peripheral hormone resides.

Radioiodine in peripheral tissues

When radioiodide in tracer dosage is administered to an organism, it is rapidly distributed throughout the interstitial fluids of the body. Within a short time the radioactivity of body water reaches a maximum intensity; and this concentration can be shown to represent ionized iodine almost exclusively. From this time onward, as illustrated in Figure 3, the "peak" activity in tissues like muscle declines continuously (22, 23) because of several phenomena:

a) Physical decay due to disintegration of unstable atoms occurs in accordance with the established half-life of the isotope involved. In the case of I^{131} this half-life is approximately eight days.

b) Iodide is excreted in the urine and other excretions so that the radioiodide is drained off progressively.

c) Some of the radioiodide becomes organically bound. Whether this binding can occur immediately in the muscle or *only* after the radioiodide has traversed the thyroid gland is part of our problem.

d) The organically bound iodine is degraded continuously in the course of natural tissue metabolism. In conducting such an experiment it is important to avoid two pitfalls. If a sample possessing very high specific radioactivity is used, the radiant energy thereof may cause morphologic or functional toxic changes which transform the experiment from a physiologic to a pharmacologic (or even pathologic) status. Likewise, if the diet or other ingesta contain too much inert iodide, this stable isotope I^{127}

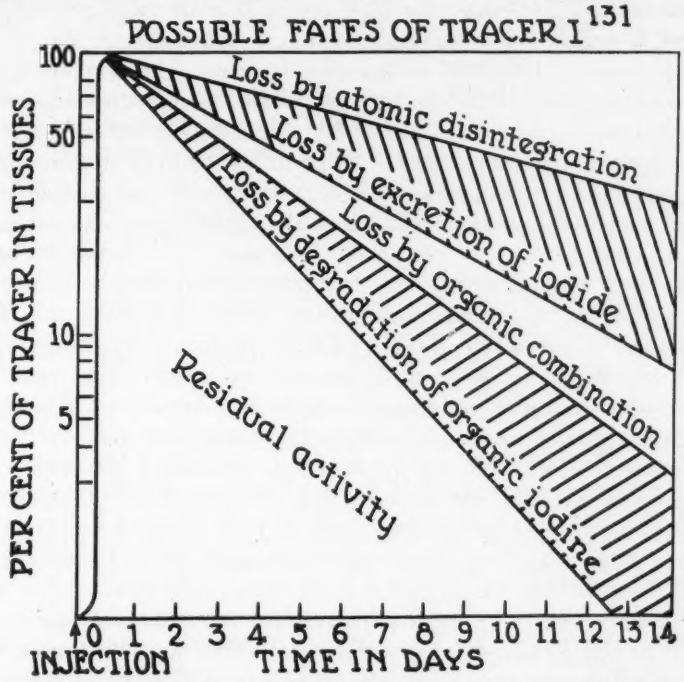
POSSIBLE FATES OF TRACER I 131
100
50
10
5
PER CENT OF TRACER IN TISSUES
Loss by atomic disintegration
Loss by excretion of iodide
Loss by organic combination
Loss by degradation of organic iodine
Residual activity
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
INJECTION
TIME IN DAYS

FIG. 3. Idealized diagram illustrating the concentration of radioiodine in a peripheral tissue following a single injection of a tracer dose. Zero time is measured from the virtual maximal concentration, reached after uniform distribution of the tracer; but this peak is slightly obscured by early excretion of the tracer.

will act as a carrier when distributed through the body. In the last instance, the experiment will then reflect the fate of the carrier rather than the natural trend of iodine metabolism.

As shown in Table 6, when radioiodide was administered to rats, the activity in skeletal muscle *at first* was found almost exclusively in the inorganic fraction. During the first days, however, tracer iodine appeared in the protein precipitate from plasma—even although control plasma *in vitro* did not show this effect. After reaching an early "peak" concentration

TABLE 6. RADIOIODINE, I^{131}, IN PERIPHERAL TISSUES. FEMALE RATS, WEIGHING APPROXIMATELY 200 GRAMS, INJECTED INTRAPERITONEALLY WITH (A) 9.0 μc. I^{131} AND (B) 7.0 μc. I^{131}

Rat number	Averaged weight (Gm.)	Time sacrificed after injection of tracer (hours)	Radioactivity			
			Muscle		Serum	
			Organic Iodine	Inorganic Iodine	Organic Iodine	Inorganic Iodine
			% $\times 10^2$ of original tracer per 100 Gm.	% $\times 10^2$ of original tracer per 100 Gm.	% $\times 10^2$ of original tracer per 100 ml.	% $\times 10^2$ of original tracer per 100 ml.
A-0, 9.0 μc.	—	0	0	0	0	0
A-1	189	3	0	438	184	300+
A-1′	185	24	0	[42]	530	350
A-2	189	48	88	170	519	154
A-3		72	30	30	—	—
A-4	190	96	36	30	112	80
A-5		120	7	23	125	32
B-0, 7.0 μc.	—	0	0	0	0	0
B-1	160, 176 → 168	4	68	1409	92	7416
B-2						
B-3	184, 177 → 181	24	38	60	163	302
B-4						
B-5	180, 180 → 180	48	0	24	199	105
B-6						
B-7	189, 180 → 185	72	0	0	159	75
B-8						
B-9	152, 164 → 158	96	0	0	129	57
B-10						
B-11	188, 170 → 179	120	0	0	86	50
B-12						

the inorganic tracer in both serum and muscle declined steadily. In all measurements shown in Table 6, appropriate corrections were made a) for atomic decay, b) for geometry of radiation and c) for internal absorption. Therefore, the changes in concentration (with time) shown in the table reflect purely fluctuations of metabolic origin, within the experimental error.

DISCUSSION

After administering radioactive thyroxine, LeBlond and his associates (24, 25, 26) have produced radioautographs of the inherent iodine located in peripheral tissues, notably in the pancreas and intestine. Such interesting studies raise the problem as to chemical mechanisms by which the hormone is fixed in cells. The techniques used in the present communication are essentially those described by Marinelli (22). The general chemical preparation of the tissue resembled that applied to the thyroid gland by Hertz, Roberts and Salter (27) and by Hamilton, Soley and their collaborators (28).

It should be noted, however, that the present studies deal with concentrations of *stable* iodine several thousand times less than those present in the thyroid gland, as described by Rawson in studies of the glandular physiology (29) or by Chapman and Evans (30) in connection with the therapy of clinical hyperthyroidism. Because the turnover rate of iodine is of great interest physiologically, as pointed out by Salter (16) collateral studies of stable iodine (naturally occurring) have been combined with measurements of radioactivity. For example, the data for *changing* radioactivity shown in Table 6 were collected from animals whose natural organically bound iodine remained within the normal range. Therefore, each of the values may be divided by a constant integer to determine the relative rate at which the peripheral hormone is "turning over" in the course of metabolism. The role played by the thyroid gland in this phenomenon is the subject of another study, but at the moment it should be noted that the dose of tracer used (9.0 μc of I^{131} per 200-gram rat) produces no obvious damage to the thyroid gland, as studied histologically.

The necessity for separating the organic from the inorganic iodine, both in serum and in tissues, has been emphasized by Salter (16). Indeed, several years ago Perkin (31) added Salter's preparation of thyroid proteose to serum and showed that it behaved like naturally circulating hormone, whereas thyroxine did not. This point has been emphasized also by McClendon (18). In the present studies the preparation of separate protein fractions from muscle, each containing different concentrations of bound iodine, has permitted similar experiments in the case of a peripheral tissue. The simple biochemical fractionation suggests that the peripheral hormone is closely allied with muscle proteins. The tracer studies show that

in muscle, as in plasma, the *inorganic iodide* builds up first. Later, radioactive *organic* iodine appears.

With respect to the turnover rate of serum hormonal iodine, Taurog, Chaikoff and Entenman (32) found that in the dog, the total amount in the circulating plasma was renewed every six hours. In a series of data for rats not reported in Table 6 it appeared that the radioactivity contained in 100 ml. of serum declined from 4.7 to 1.2 per cent of the tracer in two days. This indicates that the concentration was halved in one day, even though the concentration of stable isotope remained steady at nearly 3 micrograms per 100 ml. Thus, if the rat contained 10 cc. of serum, a renewal of 0.15 microgram of serum iodine would occur in one day.

Only very gross conclusions are suggested by the meager data as to the rate of turnover of the peripheral hormone at a normal level of metabolic rate. The normal 200-gram rat comprises about 100 grams of muscle (33). As shown in Table 6, muscle organic iodine lags behind inorganic. In a series of data for rats not reported in Table 6, this mass of muscle in 72 hours showed a change in relative concentration of tracer from 0.90 to 0.09 per cent of the tracer administered. During this time, the organic iodine in the 100-gram muscle mass was about 3 micrograms. In other words, possibly 2.7 micrograms of organic iodine per 100 grams of muscle was degraded in three days—or 0.9 microgram per day. On this basis the whole rat (200 grams) would have utilized approximately 1 to 2 micrograms of hormonal iodine per day. Several different procedures for estimating thyroxine utilization have been reviewed by Schultze and Turner (34). Astwood and Bissell (35) have applied thiouracil block of the gland to estimate the daily consumption of hormone and by this entirely independent method have arrived at an estimate approximating 5 micrograms in five days. Synthetic chemical agents which block the function of the organic iodine compound in peripheral tissues have been studied also by Winzler and Frieden (36). Much more investigation will be required, however, before final conclusions can be drawn as to the role of fixed iodine in peripheral tissues.

Interpretation of isotope measurements

Although the literature already contains many observations with tracer iodine, it is very difficult for a physiologist to ascertain the significance of such data. Many authors assume that labelled iodine directly measures newly made hormone. Actually, the newly made hormone, H, is dissipated progressively in part, along with the old hormone, as time passes. Therefore, if the rat's serum "hormonal" iodine is maintained at 2.5 micrograms per cent,

$$1) \qquad \frac{dH}{dt} - \left(1 - \frac{L}{2.5}\right) = dL$$

where L is the concentration of accumulated labelled iodine which has escaped metabolic degradation. Hence, at any time, t_0, after the injection of tracer,

2) $$H_{t_0} = -2.5\ ln\left(1 - \frac{L_{t_0}}{2.5}\right)$$

by integration.

In order to solve this equation, the investigator must evaluate the concentration of labelled iodine, L. This he does by measuring the radioactivity, A, of the "hormonal" (protein-bound) iodine. Unfortunately, the specific radioactivity of the bodily supply of iodide (from which new H is made) is declining steadily. This decline has nothing to do with the physical decay of atoms; but is caused by the progressive excretion of radioiodide, as indicated in Figure 3. Therefore, to correct for the progressive *metabolic* weakening of the specific activity, σ, of the body's iodide reserves, the investigator must employ two other equations, which relate to labelled iodine:

3) $$\sigma = \frac{d\sigma}{-K dt} \quad \text{or} \quad \frac{-d\ \log \sigma}{dt} = K$$

and

4) $$\frac{dL}{dt} = \frac{dA}{\sigma dt} = \frac{1}{K_0}\, e^{k_1 t}\, \frac{dA}{dt}$$

Because σ and A can be measured at stated times, it is possible to compute L_{t_0}, the concentration of labelled organic iodine at the time, t_0 (e.g., 48 hours), after the injection of the tracer. This value is then substituted into equation No. 2.

Without this mathematical approach, the physiologic meaning of many tracer measurements remains obscure.

SUMMARY

As found with the blood plasma, peripheral tissues contain organically bound iodine which rises and falls with fluctuations in thyroid activity and with the resulting changes in basal metabolic rate. In muscle, as in plasma, this organic iodine is unevenly distributed among the several protein fractions. In plasma and in skeletal muscle, too, the native organic iodine behaves differently than does free thyroxine during the acetone-precipitation of proteins. After the administration of radioiodide in tracer dosage, the activity resides at first chiefly in the inorganic fraction but, later, part of the activity becomes attached to protein.

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THE INCREASED PROTEIN-BOUND BLOOD IODINE IN PATIENTS WITH HYPERTHYROIDISM*†

ROY E. SWENSON, M.D. AND GEORGE M. CURTIS, M.D.

From The Department of Research Surgery, The Ohio State University, Columbus, Ohio

GLEY and Bourcet (1) dialyzed dog serum as early as 1900 and concluded that the serum iodine, for the most part, existed in protein combination. Veil and Sturm (2) separated the whole-blood iodine into alcohol-soluble and alcohol-insoluble fractions. Holst (3) and Lunde (4) reported that the alcohol-insoluble fraction was related to the thyroid hormone and that its concentration was proportional to the basal metabolic rate. Reports, confirming the finding that the PBI[1] is elevated in patients with hyperthyroidism, followed (5, 6, 7, 8, 9, 10).

Salter and Bassett (11) divided the plasma or serum iodine into inorganic, thyroxine-like and diiodotyrosine-like fractions, the latter two composing 30 to 40 per cent and 60 to 70 per cent of the protein-bound fraction respectively. The PBI concentration of plasma or serum normally averages approximately 5.0 micrograms per cent when the proteins are precipitated by heat and acetic acid (9), the Somogyi reagents (12, 13) or methanol followed by acetone washing (8).

In central Ohio, the average whole-blood iodine concentration is 4.2 ± 1.2 micrograms per cent (14, 15, 16). This value agrees with that determined by other investigators (17, 18, 19, 20). The average level for the acetone-insoluble fraction is 0.88 ± 0.2 micrograms per cent; it is decreased significantly in patients with hypothyroidism and elevated in those with hyperthyroidism (21, 22). This fraction composes 20 to 30 per cent of the whole-blood iodine. This is lower than the 60 to 70 per cent figure reported by others (9, 10, 11, 12, 13) as the protein-bound iodine of serum or plasma.

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[1] PBI = Protein-bound Blood Iodine.

This article will be included in the bound volume of the "Transactions of the American Goiter Association," published by Charles C Thomas, Publisher, which will be available for sale early in 1949.

TABLE 1. ACETONE-FRACTIONATION OF THE SERUM IODINE

5.0 cc. aliquot of serum	Mcg.% iodine in insoluble fraction	
1.	4.91	
2.	4.88	
3.	5.1	
15.0 cc. aliquot of serum		
1.	5.08	
2.	4.9	Average = 4.97
3.	5.0	

The average value for the serum-precipitable iodine as determined following acetone precipitation, closely agrees with that determined by other investigators using other precipitating agents.

Nature of the Acetone-Insoluble Fraction of the Blood Iodine

Normal human serum was collected, pooled, fractionated with acetone and analyzed for iodine (14, 15). Table 1 summarizes the data. The acetone-insoluble iodine closely approximates the value reported by others using various methods of protein precipitation (9, 10, 11, 12, 13). It is likely that the differences between the PBI as determined by acetone fractionation and that reported by others for serum or plasma are more superficial than

TABLE 2. CHANGES IN THE FRACTIONS FOLLOWING THE ADDITION OF NORMAL HUMAN THYROGLOBULIN TO POOLED NORMAL HUMAN SERUM. 8.726 ± .25 MICROGRAMS OF IODINE ADDED AS THYROGLOBULIN

(Experimental error of the method ±3 per cent)

Micrograms of iodine in 5.0 cc. of control serum			Micrograms of iodine in 5.0 cc. of serum plus thyroglobulin			Micrograms increase in the 2 fractions		Per cent recovery in the insoluble fraction
Total	Acetone-soluble	Acetone-insoluble	Total	Acetone-soluble	Acetone-insoluble	Acetone-soluble	Acetone-insoluble	
5.45 ± .1*	5.3 ± .1	0.245	14.16	5.793	8.367	0.493	8.122	93.5%
Micrograms of iodine in 20.0 cc. of control serum			Micrograms of iodine in 15.0 cc. of serum plus 5 cc. of serum containing the thyroglobulin aliquot			Micrograms increase in the 2 fractions		Per cent recovery in the insoluble fraction
Total	Acetone-soluble	Acetone-insoluble	Total	Acetone-soluble	Acetone-insoluble	Acetone-soluble	Acetone-insoluble	
23.57	22.59	0.98	31.908	22.0	9.908	0.00	8.928	100%

93.5–100 per cent of iodine added as thyroglobulin is recovered in the protein coagulum following acetone precipitation.

* Each figure in the table is an average of 3 determinations, made after 50 determinations showing a similar recovery.

real and are due to inherent differences between whole-blood and serum or plasma.

Since it is unlikely that thyroxine or diiodotyrosine occur free in the blood, recovery experiments following the addition of these substances to whole-blood or serum may be misleading. Consequently a normally occurring iodoprotein, normal human thyroglobulin, was secured from Dr. J. F. McClendon[2] and was quantitatively added to serum which was subsequently fractionated with acetone and then analyzed for iodine. Table 2 summarizes the data. From 93.5 to 100 per cent of the iodine added as

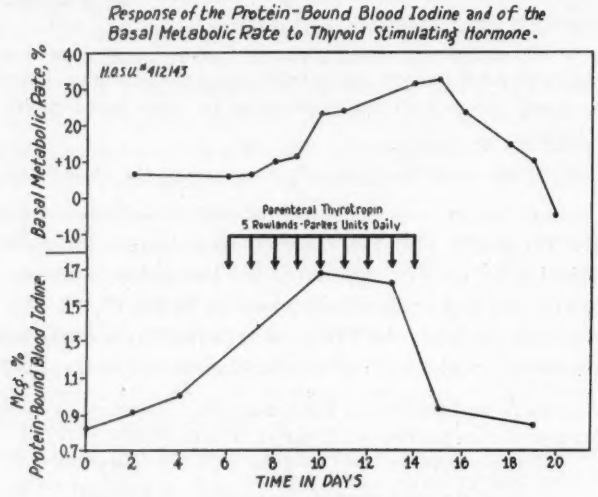

FIG. 1

normal human thyroglobulin is recovered in the protein coagulum. Consequently, the acetone-insoluble fraction appears to be thyroglobulin-like in nature.

The Acetone-Insoluble Fraction of the Blood Iodine as an Index of Thyroid Function

Two qualifications necessary for accepting a blood iodine fraction as an index of thyroid function are that:

1. It should increase along with the basal metabolic rate following the administration of thyrotropic hormone; and that
2. It should decrease along with the basal metabolic rate following the blockade of thyroid function by the goitrogenic drugs.

[2] We wish to thank Dr. J. F. McClendon for this courtesy.

Figure 1 illustrates the response of this fraction of the blood iodine to thyrotropic hormone stimulation in man. The rise and fall along with the basal metabolic rate are in accordance with the present concept of initial stimulation, followed by a refractory period during which the metabolism decreases even though thyrotropic stimulation is continued (23, 24). Figure 2 illustrates the response of this fraction to the administration of cyclopropylthiouracil, 200 mg. daily, in primary hyperthyroidism. Each succeeding cycle of activity of the disease is separated from its predecessor by

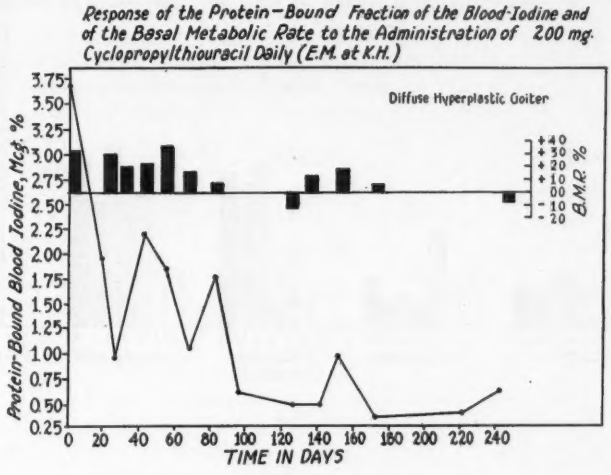
Response of the Protein-Bound Fraction of the Blood-Iodine and of the Basal Metabolic Rate to the Administration of 200 mg. Cyclopropylthiouracil Daily (E.M. at K.H.)
Diffuse Hyperplastic Goiter
Protein-Bound Blood Iodine, Mcg. %
B.M.R. %
TIME IN DAYS

FIG. 2

a great interval of time while the BMR and PBI are lower with each succeeding cycle.

The Iodination of Protein and the Protein-Bound Blood Iodine

Many investigators have studied the iodoproteins (25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37). Thyroxine has been isolated from iodinated casein, and optimum results were secured when 4 atoms of iodine per molecule of tyrosine were added (38, 39, 40). Similarly, thyroxine-like and diiodotyrosine-like peptides have been secured from iodoproteins prepared from serum proteins *in vitro* (32). The Schärdinger enzyme may be intimately concerned with such biologic iodinations (41).

Following iodine therapy of hyperthyroidism, Salter noted "spurious" elevation of the protein-bound serum iodine but thought it a chemical artefact; however, he reproduced the phenomenon *in vitro* (11). In order to determine the relation of protein iodination to the PBI, varying amounts

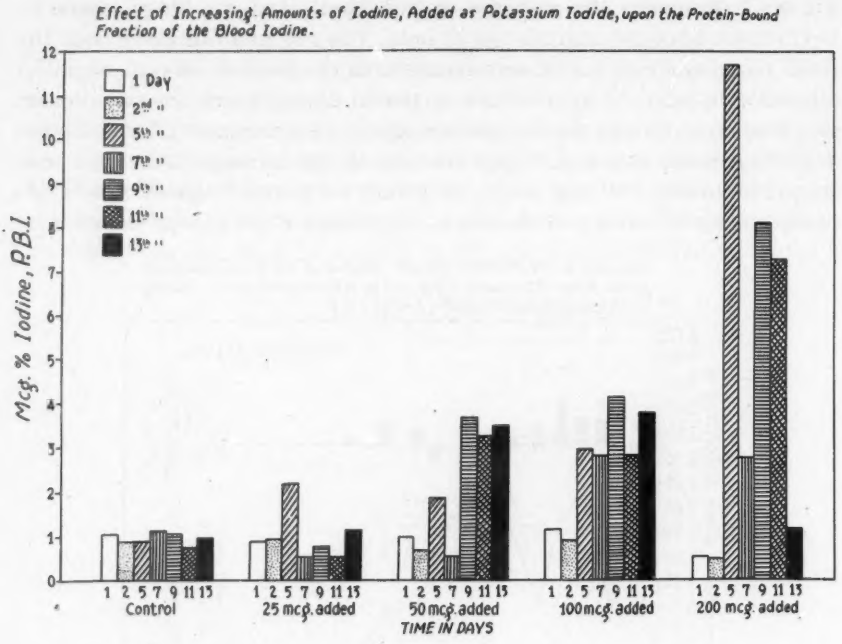
Effect of Increasing Amounts of Iodine, Added as Potassium Iodide, upon the Protein-Bound Fraction of the Blood Iodine.
1 Day
2nd "
5th "
7th "
9th "
11th "
13th "
Mcg. % Iodine, P.B.I.
0 1 2 3 4 5 6 7 8 9 10 11 12
1 2 5 7 9 11 13
Control
25 mcg. added
50 mcg. added
100 mcg. added
200 mcg. added
TIME IN DAYS

FIG. 3

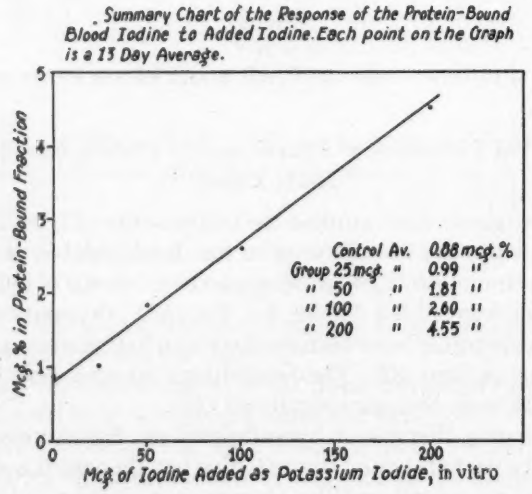
Summary Chart of the Response of the Protein-Bound Blood Iodine to Added Iodine. Each point on the Graph is a 13 Day Average.
Mcg. % in Protein-Bound Fraction
0 1 2 3 4 5
0 50 100 150 200
Mcg. of Iodine Added as Potassium Iodide, in vitro
Control Av. 0.88 mcg. %
Group 25 mcg. " 0.99 "
" 50 " 1.81 "
" 100 " 2.60 "
" 200 " 4.55 "

FIG. 4

of potassium iodide were added to human whole-blood *in vitro*, and fractionation with acetone was carried out after varying intervals of time.

Figure 3 demonstrates that but little iodination of protein occurs following this procedure until the total iodine concentration reaches 100 micrograms per cent; further a striking increase in the protein-bound fraction occurs on the fifth day, strongly suggestive that the reaction at first proceeds according to a "lag phase" and then by a "log phase." This lends support to the premise (41) that certain enzymes play a role in such iodinations. Figure 4 is a summary graph of determinations made over a 13-day

TABLE 3. RATIO OF THE INCREASE IN THE PROTEIN-BOUND BLOOD IODINE TO THE AMOUNT OF POTASSIUM IODIDE ADDED TO WHOLE BLOOD

Mcg. per cent iodine added	Calculation	Ratio
50	$\frac{0.11}{50} \times 100$	0.22
100	$\frac{0.93}{100} \times 100$	0.93
200	$\frac{1.72}{200} \times 100$	0.86
400	$\frac{3.67}{400} \times 100$	0.90

period, and suggests a positive correlation between the concentration of iodine present, the time it has been present and the iodination of protein.

Using the formula:

$$\frac{\text{Mcg. per cent increase in PBI over control PBI}}{\text{Mcg. per cent present in the sample}} \times 100 = R$$

it is possible to determine whether the iodination proceeds irrespective of the quantity of iodine added as iodide. Table 3 summarizes these data. This suggests that within the limits of experimental error, the increase of the PBI following the addition of potassium iodide is proportional to the added iodine in a ratio of 0.9 plus per cent, when the concentration of such iodine exceeds 50 micrograms per cent.

When the average response of the PBI to iodine therapy in primary hyperthyroidism is compared to that of toxic nodular goiter, a striking differ-

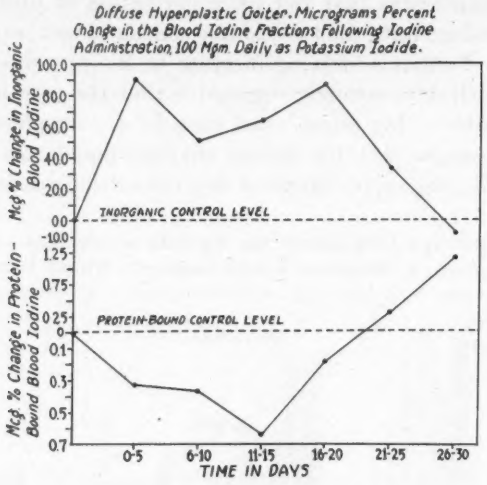

FIG. 5

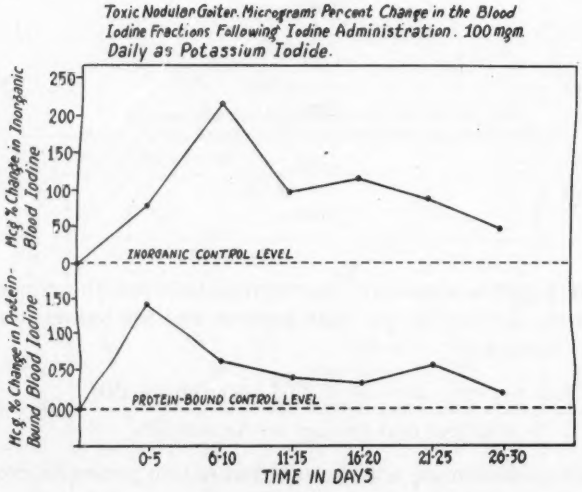

FIG. 6

ence is apparent. The average response in primary hyperthyroidism (Fig. 5) is in accordance with the present accepted concepts of the mechanisms of the iodine-induced remissions from hyperthyroidism. It is likely that

TABLE 4. DIFFUSE HYPERPLASTIC GOITER. ELEVATION OF THE PROTEIN-BOUND BLOOD IODINE AND DECREASE IN THE BASAL METABOLIC RATE, FOLLOWING THE ADMINISTRATION OF POTASSIUM IODIDE

Days of iodine therapy	Inorganic fraction, mcg. %	Protein-bound fraction, mcg. %	Basal metabolic rate, %
0	2.62	1.10	+48
4	44.40	3.04	+44
7	55.00	1.86	—
8	66.80	1.86	+40
0	9.63	1.69	+57
24	17.35	3.72	+12
0	6.50	1.40	+44
38	65.50	7.20	+26
0	4.22	1.77	+48
7	3.56	1.36	+37
17	2.96	2.54	+18
21	1.10	3.72	+15
29	1.14	11.80	+ 8
0	5.66	1.52	+74
3	67.30	1.86	+70
7	52.90	1.86	+54
0	2.96	1.94	+32
15	942.40	3.90	− 3
0	8.72	1.94	+50
31	48.10	21.20	+22
0	9.39	2.54	+54
19	318.54	3.80	+28
0	7.62	2.04	+55
18	31.78	3.98	+40
0	9.48	2.45	+66
9	41.38	3.30	+48
16	232.93	3.12	+43

iodine inactivates thyrotropin (42) and temporarily inactivates enzyme systems releasing thyroid hormone from the thyroid follicles (43, 44). The average response in toxic nodular goiter (Fig. 6) mirrors the level of the inorganic blood iodine, and is similar to the response noted following the

TABLE 5. TOXIC NODULAR GOITER. ELEVATION OF THE PROTEIN-BOUND BLOOD IODINE AND DECREASE IN THE BASAL METABOLIC RATE, FOLLOWING THE ADMINISTRATION OF POTASSIUM IODIDE

Days of iodine therapy	Inorganic fraction mcg. %	Protein-bound fraction, mcg. %	Basal metabolic rate, %
0	6.94	1.94	+30
23	22.84	3.98	+10
0	12.18	2.36	+57
7	508.00	3.14	+48
13	139.50	3.14	+31
0	5.5	.94	+36
7	180.0	5.08	+29
0	5.84	.94	+38
18	18.30	10.66	+12
0	5.82	1.40	+30
25	24.78	2.28	+23
0	7.73	5.83	+55
6	136.2	7.36	+40
0	6.94	1.94	+30
23	22.84	3.98	+10
0	7.18	1.94	+50
5	5.66	2.88	+39
10	7.95	2.20	+35
0	5.92	9.82	+77
18	926.00	11.8	+29
0	5.58	1.01	+36
20	212.82	3.04	+ 6

in vitro addition of potassium iodide to blood. It is difficult to explain these variances unless fundamental differences in tyrosine metabolism, diiodotyrosine and thyroxine turnover and thyroglobulin metabolism exist between the two types of hyperthyroidism.

Taurog and Chaikoff (45) have reported that following iodine administration to rats, the level of the protein-bound plasma iodine is dependent upon the thyroxine content of the thyroid gland, and is limited by the capacity of the thyroid to produce thyroxine. Our data indicate that the iodination

of protein may occur within the blood itself. Such iodination is generally attributed to the preferential iodination of tyrosine. Such a reaction may be physiologically important in the induction of remissions from hyperthyroidism.

If the circulating thyroid hormone is composed of diiodotyrosine and thyroxine, it would be expected that an increase of one or both in the circulating blood would exert an inhibitory influence upon the production of thyrotropin by the anterior pituitary. Thus it would be expected that if the iodination of protein occurred *in vivo* following the administration of iodine, the PBI would rise, mirroring the iodination of tyrosine, while the basal metabolic rate continued to fall. Tables 4 and 5 demonstrate this phenomenon in 10 patients with primary hyperthyroidism and in 10 with the hyperthyroidism associated with nodular goiter. The true significance of this phenomenon in the iodine-induced remissions from hyperthyroidism remains to be evaluated.

The Protein-Bound Blood Iodine in Patients with Nodular Goiter

A comparison of the PBI in 178 untreated, uncomplicated instances of nontoxic nodular goiter with that in a similar group of 114 patients with

TABLE 6. NONTOXIC NODULAR GOITER. PERCENTAL DISTRIBUTION OF THE BASAL METABOLIC RATE WITHIN THE 2 STANDARD DEVIATION* LIMITS: ITS CORRELATION TO THE PROTEIN-BOUND BLOOD IODINE CONCENTRATION AND TO THE SYMPTOMATOLOGY PRESENTED

No. of patients	Basal Metabolic rate, %							Summary of findings	
	Range	Below −21	−21 to −13	−13 to −5	−5 to +3	+3 to +11	Above +11		
178	−26 to +24	2.8	9.5	20.7	23.6	25.8	17.4	Per cent of all patients with basal metabolic rates of +3% or above	43.2
Per cent of each BMR group with one or more symptoms of hyperthyroidism		40.0	60.0	54.0	64.3	73.9	80.6	Per cent of 178 patients with one or more symptoms of hyperthyroidism	66.8
Protein-bound blood iodine concentrations in micrograms per 100 cc.		0.51	0.77	0.86	0.88	1.21	1.32	Per cent of patients with one or more symptoms of hyperthyroidism 119 and protein-bound blood iodine concentration in excess of 0.88 micrograms per 100 cc.	70.6
Per cent of each BMR group having protein-bound blood iodine concentrations above 0.88 micrograms per 100 cc.		0.0	29.4	35.1	52.3	69.6	80.6	Per cent of 178 patients with protein-bound blood iodine concentrations in excess of 0.88 micrograms per 100 cc.	55.0

* (Average normal B.M.R. = minus 5 ± 8%. Average Protein-bound Blood Iodine = 0.88 ± 0.2 mcg. per cent).

clinical hyperthyroidism is most instructive. Table 6 presents a summary of the former, and Table 7 summarizes the latter. Figure 7 portrays the progressive, parallel linear relationship of the BMR to the PBI in each group. Particular attention is called to the groups having BMR's between plus 3 and plus 11 per cent; the average PBI concentrations here were 1.2

TABLE 7. TOXIC NODULAR GOITER. DISTRIBUTION OF PATIENTS ACCORDING TO STANDARD DEVIATIONS* OF THE BASAL METABOLIC RATE AND THE PROTEIN-BOUND BLOOD IODINE

	Basal metabolic rate, %							
	+3 to +11	+11 to +19	+19 to +27	+27 to +35	+35 to +43	+43 to +59	Above +59	
PBI, mcg. %	1.27	1.65	1.85	1.94	2.16	2.46	3.33	
No. of patients in each BMR group	14	12	16	26	17	19	10	Total patients 114
Per cent of each group with PBI levels above 0.88 mcg. %	85.7	100	100	100	100	100	100	Total patients with PBI above 0.88 mcg. % = 105, or 91.2%
Per cent of each group with symptoms suggestive of hyperthyroidism	93	91	100	96.1	100	84.2	90	Per cent of total patients with one or more symptoms suggestive of hyperthyroidism = 93.8
Per cent of each group with PBI levels above 1.2 mcg. %, the upper limit of normal	57.1	100	93.7	92.3	90	100	100	Total patients with PBI levels above 1.08 mcg. % = 102, or 89.4%

* (Average normal B.M.R. = minus $5 \pm 8\%$. Average Protein-bound Blood Iodine $= 0.88 \pm 0.2$ mcg. per cent.)

mcg. per cent for the clinically nontoxic group and 1.27 mcg. per cent for the clinically hyperthyroid group. The difference is less than that of the associated experimental error.

In the nontoxic group 70.6 per cent of those with symptoms had elevated PBI levels, while only 47 per cent had elevations of the basal metabolic rate. The percental incidence of symptoms suggestive of hyperthyroidism was found to be significantly correlated with a BMR of plus 3 per

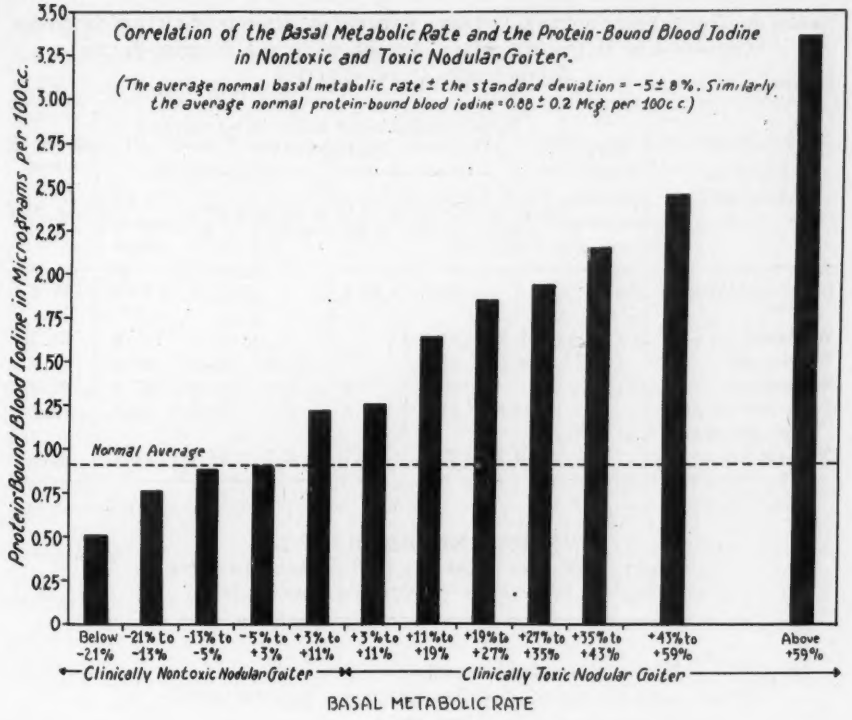

FIG. 7

cent or above (Table 8) and with a PBI of 1.2 mcg. per cent or above (Table 9). In this group the symptoms suggestive of hyperthyroidism, listed according to their positive correlation to an elevated PBI (Fig. 8)

TABLE 8. NONTOXIC NODULAR GOITER. PERCENTAL DISTRIBUTION OF SYMPTOMS SUGGESTIVE OF HYPERTHYROIDISM, CORRELATED TO THE BASAL METABOLIC RATE

Symptom presented	Total times symptom occurred	Basal metabolic rate, %						Total % above +3
		Below −21	−21 to −13	−13 to −5	−5 to +3	+3 to +11	Above +11	
Emotionalinstability	9		33.3	22.2		33.3	11.1	44.4
Weakness	19			21.0	31.6	31.6	15.7	47.3
Weight loss	25		4.0	16.0	16.0	36.0	28.0	64.0
Palpitation	29		13.8	3.4	24.1	37.9	20.7	58.6
Pulse rate of 90 or more per minute	42		9.5	14.3	14.3	23.8	38.1	61.9
Nervousness	73	4.1	11.0	17.8	21.9	26.0	19.2	45.2

TABLE 9. NONTOXIC NODULAR GOITER. PERCENTAL DISTRIBUTION OF SYMPTOMS SUGGESTIVE OF HYPERTHYROIDISM, ACCORDING TO THE PROTEIN-BOUND BLOOD IODINE CONCENTRATION

Symptom presented	Total times symptom occurred	Protein-bound blood iodine in micrograms per 100 cc.						Total % above 0.88 mcg./100 cc.
		0.26 to 0.50	0.51 to 0.76	0.77 to 0.85	0.86 to 0.88	0.89 to 1.21	1.22 and above	
Emotional instability	9		33.3	22.2		33.3	11.1	44.4
Weakness	19	5.2	31.6			31.6	31.6	63.2
Weight loss	25	8.0	24.0		4.0	28.0	36.0	64.0
Palpitation	29		20.7	3.5	6.8	31.0	37.8	68.8
Pulse rate of 90 or more per minute	42	4.8	11.9	4.8	2.4	28.5	47.6	76.1
Nervousness	73	8.2	20.5	4.1	5.5	27.4	34.2	61.6

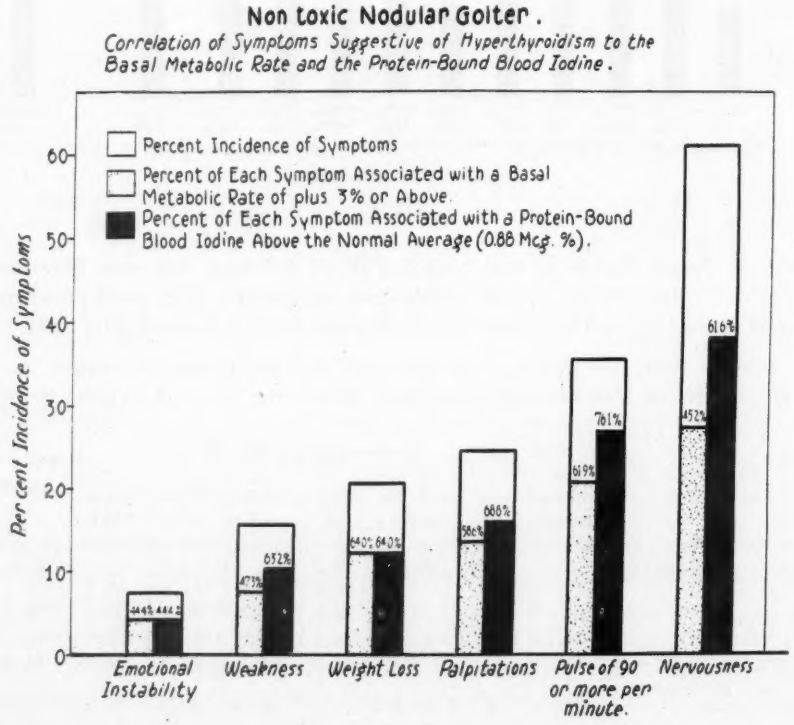

FIG. 8

are: a pulse of 90 or more per minute, palpitation, weight loss, weakness, nervousness and emotional instability.

Of those with nodular goiter and hyperthyroidism, 93.8 per cent presented

TABLE 10. TOXIC NODULAR GOITER. PERCENTAL DISTRIBUTION OF SYMPTOMS SUGGESTIVE OF HYPERTHYROIDISM, ACCORDING TO THE BASAL METABOLIC RATE

Symptoms presented	No. of times symptoms occurred	Basal metabolic rate, %						
		+3 to +11	+11 to +19	+19 to +27	+27 to +35	+35 to +43	+43 to +59	Above +59
Pulse rate of 90 or more per min.	71	9.9	8.5	14.0	23.9	18.3	11.3	14.0
Palpitation	64	10.9	10.9	9.4	25.0	15.6	18.8	9.4
Weight loss	67	9.0	13.4	14.9	23.9	11.9	11.9	14.9
Weakness	35	5.7	8.6	14.3	11.4	20.0	22.9	17.1
Emotional instability	20	15.0	20.0		20.0	5.0	20.0	20.0
Nervousness	74	6.8	14.9	13.5	24.3	13.5	13.5	13.5
Tremor	27	3.7	18.5	7.4	14.8	29.6	14.8	11.1
Sensitivity to heat	19	21.0	5.3	10.5	36.8	5.3	15.8	5.3

TABLE 11. TOXIC NODULAR GOITER. PERCENTAL DISTRIBUTION OF THE SYMPTOMS, ACCORDING TO THE PROTEIN-BOUND BLOOD IODINE

Symptoms presented	No. of times symptoms occurred	Protein-bound blood iodine, mcg. %						
		1.27	1.27 to 1.65	1.65 to 1.85	1.85 to 1.94	1.94 to 2.16	2.16 to 2.46	2.46+
Pulse rate of 90 or more per min.	71	22.5	14.1	15.5	7.0	12.7	2.8	25.4
Palpitation	64	18.8	18.8	14.0	7.8	9.4	3.1	28.1
Weight loss	67	19.4	14.9	14.9	6.0	10.4	4.5	29.9
Weakness	35	17.2	14.3	8.6	11.4	11.4	5.7	31.4
Emotional instability	20	35.0	10.0	5.0	10.0	5.0	10.0	25.0
Nervousness	74	18.9	20.3	12.2	8.1	9.5	4.0	27.0
Tremor	27	29.6	11.1	14.8	14.8		3.7	25.9
Sensitivity to heat	19	15.8	26.3	21.0	15.8	5.3		15.8

one or more of the symptoms previously listed. The percental incidence is correlated to the BMR in Table 10 and to the PBI in Table 11. Approximately 36 per cent of the symptomatology exhibited was associated with PBI concentrations of from 1.27 to 1.65 mcg. per cent; and strongly suggests

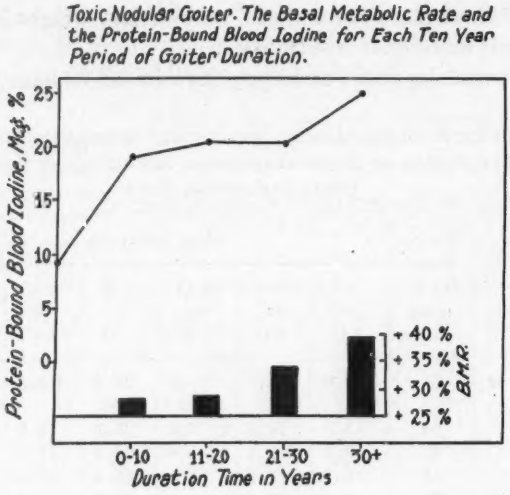

FIG. 9

TABLE 12. TOXIC NODULAR GOITER. RELATIONSHIP BETWEEN DURATION OF GOITER, THE BASAL METABOLIC RATE AND THE PROTEIN-BOUND BLOOD IODINE

Duration of goiter in decades	Average duration in in years	Average basal metabolic rate %	Average protein-bound blood iodine, mcg. %	No. of patients
0– 5	1.86	+28.3	1.94	32
6–10	8.00	+28.3	1.55	8
0–10	3.09	+28.3	1.86	40
11–15	13.5	+31.0	2.25	13
16–20	18.6	+26.7	1.94	16
11-20	16.3	+28.7	2.08	29
21–25	24.8	+32.1	2.07	7
26–30	30.0	+34.5	2.09	8
21–30	27.6	+33.4	2.08	15
over 30	39	+39.5	2.50	18

a continuation of the significant percental incidence of symptoms noted for that in nontoxic nodular goiter to be associated with a PBI concentration of 1.2 mcg. per cent or above.

Figure 9 graphically illustrates the relation of goiter duration to the BMR and the PBI in nodular goiter with hyperthyroidism. Table 12 summarizes the latter group. In the nontoxic group the PBI never falls below the normal average, and is most elevated between the 10 and 20-year period of duration (cf. (22) fig. 2). In the hyperthyroid group, the 0–10 and 10–20 year averages exhibit a constant rise. It is likely that the longer goiter is present, the more frequently elevations of the BMR and PBI occur.

These findings suggest that for the region of central Ohio, persons with nodular goiters and PBI concentrations of 1.2 mcg. per cent or above and 1) symptoms suggestive of hyperthyroidism, or 2) basal metabolic rates of plus 3 per cent or above, are potentially hyperthyroid. It follows that the optimum time for nodular goiters to be removed would appear to be at the time when hyperthyroidism is in its early or incipient stages, the criteria for which have been presented.

The Protein-Bound Blood Iodine in Diffuse Hyperplastic Goiter

In general, the PBI averages for the BMR groups are somewhat higher in primary hyperthyroidism than in nodular goiter with hyperthyroidism. Table 13 summarizes the distribution in 90 patients with untreated, uncomplicated instances of primary hyperthyroidism. Figure 10 demonstrates

TABLE 13. DIFFUSE HYPERPLASTIC GOITER. DISTRIBUTION ACCORDING TO 2 STANDARD DEVIATIONS* OF THE BASAL METABOLIC RATE AND CORRELATION TO THE PROTEIN-BOUND BLOOD IODINE

B.M.R. %	−5 to +3%	+3% to +19%	+19% to +35%	+35% to +51%	+51% to +67%	+67% to +83%	Above +83%
No. of patients in each group	4	13	22	16	14	13	8
Average PBI, mcg. %	1.53	1.96	2.07	2.10	2.98	3.15	3.38
Per cent of each group with one or more symptoms of hyperthyroidism	100	100	100	100	100	100	100

* See footnote to Table 6.

a progressive linear relation between the BMR and the PBI. As the BMR increases from between minus 5 to plus 3 per cent and on up to above plus 83 per cent, the PBI concentration progressively increases from 1.53 mcg. per cent to 3.38 mcg. per cent.

Each of the 90 patients with primary hyperthyroidism presented one or

more suggestive symptoms; this higher incidence of symptoms parallels the higher PBI noted. Table 14 summarizes the correlation of the percental incidence of symptoms with the BMR and Table 15, to the PBI.

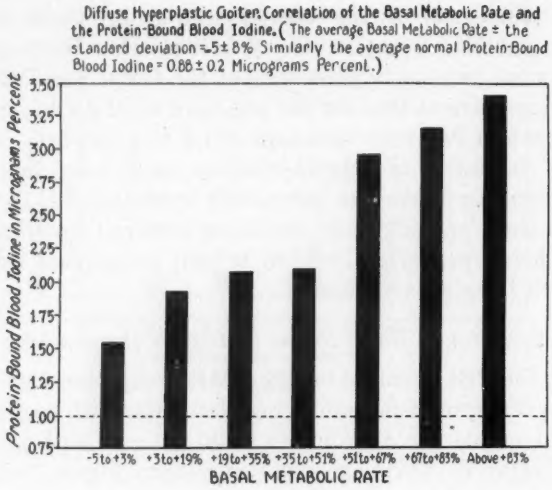

FIG. 10

TABLE 14. DIFFUSE HYPERPLASTIC GOITER. CORRELATION OF THE PERCENTAL INCIDENCE OF THE SYMPTOMATOLOGY TO THE BASAL METABOLIC RATE

Symptom presented	No. of times symptom occurred	Basal metabolic rate					
		+3 to +19%	+19 to +35%	+35 to +51%	+51 to +67%	+67 to +83%	Above +83%
Pulse of 90 or more per minute	67	10.4%	22.3%	20.9%	13.5%	19.4%	13.4%
Palpitation	50	14.0	18.0	24.0	10.0	18.0	16.0
Weight loss	55	12.7	20.0	21.8	14.5	20.0	10.9
Nervousness	69	17.4	23.1	15.9	14.5	17.4	11.5
Emotional instability	30	23.3	16.6	10.0	13.3	23.3	13.3
Tremor	27	14.8	18.5	14.8	11.1	25.9	14.8
Sensitivity to heat	26	11.5	23.1	11.5	23.1	7.6	23.1
Weakness	35	17.1	22.8	17.1	14.4	17.1	11.5

The percental incidence of symptoms is rather evenly distributed throughout the BMR range, yet the symptomatology tends to occur in 3 phases when correlated with the PBI:

TABLE 15. DIFFUSE HYPERPLASTIC GOITER. CORRELATION OF THE PERCENTAL INCIDENCE OF THE SYMPTOMATOLOGY TO THE PROTEIN-BOUND BLOOD IODINE

Symptom presented	No. times symptom occurred	Protein-bound blood iodine, mcg. %						
		1.53 to 1.96	1.96 to 2.07	2.07 to 2.10	2.10 to 2.98	2.98 to 3.15	3.15 to 3.38	Above 3.38
Pulse of 90 or more per minute	67	35.8%	4.4%	1.4%	25.4%		4.4%	28.5%
Palpitation	50	42.0	6.0		26.0		2.0	24.0
Weight loss	55	38.2	1.8	1.8	29.1		3.6	25.4
Nervousness	69	36.2	5.8	1.4	30.4		5.8	20.3
Emotional instability	30	43.3	3.3		10.0		13.3	30.0
Tremor	27	29.6			33.3		3.7	33.3
Sensitivity to heat	26	34.5	3.9		42.3			19.2
Weakness	35	31.5	5.7	2.8	25.7		5.7	28.5

1. From 1.53 to 1.96 mcg. per cent.
2. From 2.10 to 2.98 mcg. per cent.
3. Above 3.38 mcg. per cent.

SUMMARY AND CONCLUSIONS

1. Acetone fractionation of pooled human serum has revealed that approximately the same amount of iodine is retained in the protein coagulum as when other protein precipitants are used.

2. When the normally occurring iodoprotein, normal human thyroglobulin, is added to pooled, normal, human serum, and acetone fractionation then carried out, from 93.5 to 100 per cent of the iodine added as thyroglobulin is recovered in the insoluble fraction. Presumably this fraction is largely thyroglobulin-like in nature.

3. Following the addition of potassium iodide to human blood *in vitro*, iodination of protein occurs in a ratio of 0.9 plus per cent when the concentration of the added iodine exceeds 100 mcg. per cent. The striking increase in the protein-bound fraction on the fifth day following the addition of iodine strongly suggests the role of certain enzyme systems within the blood itself in the iodination of protein.

4. The average responses of the protein-bound blood iodine (PBI) to the administration of potassium iodide in patients with primary hyperthyroidism and in those with nodular goiter with hyperthyroidism are quite different. The average response in primary hyperthyroidism follows the

generally accepted conception of an initial decrease in the PBI followed by a rise, as the iodine-induced remission recedes. In nodular goiter with hyperthyroidism, the average response parallels that noted for the addition of potassium iodide to blood *in vitro*. Iodination occurs, however, in both types of hyperthyroidism. The role of the iodinated blood proteins in iodine-induced remissions from hyperthyroidism is yet to be evaluated.

5. The acetone-insoluble fraction of the blood iodine increases along with the basal metabolic rate following the administration of thyrotropin and follows the generally accepted principles of the refractory period to thyrotropin stimulation. Similarly the acetone-insoluble fraction decreases along with the basal metabolic rate following the administration of the goitrogenic drugs. Thus, the acetone-insoluble fraction of the blood iodine is a good index of thyroid function.

6. In untreated, uncomplicated nontoxic nodular goiter, the basal metabolic rate and the PBI bear a progressive, linear relation to each other. Similarly, an identical relationship exists in nodular goiter with hyperthyroidism but at a higher level. When patients with clinically toxic and clinically nontoxic nodular goiters are compared, there is considerable overlapping in clinical diagnosis when the BMR is in excess of plus 3 per cent and the PBI exceeds 1.2 mcg. per cent.

7. The symptomatology presented by patients with nontoxic and toxic nodular goiter, the similar linear relationship between the BMR and the PBI, the significant relationship of the PBI to the duration of goiter, and the overlapping diagnoses when the BMR is between plus 3 and plus 11 per cent, indicate that nodular goiter with hyperthyroidism develops as a sequel to nontoxic nodular goiter, and consequently is more apt to occur as the duration of goiter increases.

8. Analysis of the symptoms, the BMR and the PBI, suggests that the optimum time for nodular goiters with hyperthyroidism to be removed is while hyperthyroidism is in its early or incipient stages, i.e., when the BMR exceeds plus 3 per cent and the PBI exceeds 1.2 mcg. per cent.

9. In diffuse hyperplastic goiter, there is a similar linear relation between the BMR and the PBI, but at a higher level. As a consequence, the incidence of symptoms in this type of hyperthyroidism is higher. The incidence of symptoms appears to occur in three phases as the protein-bound blood iodine increases.

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ASSAY OF THYROTROPIC HORMONE IN HUMAN BLOOD

E. De ROBERTIS, M.D.*

THE discovery that under physiological conditions thyroid activity is regulated by the pituitary gland and the demonstration of a balanced interrelationship between the secretion of thyroid hormone (TH) and of thyroid-stimulating hormone (TSH) has led to the supposition that some thyroid diseases might represent alterations of these mechanisms. This hypothesis has been mentioned repeatedly in the literature since Loeb and Bassett (1) suggested that excessive secretion of thyrotropic hormone by the pituitary gland might be of etiological importance in toxic goiter, basing their premise on similarities between the changes observed in animals after the injection of TSH and the symptoms of Graves' disease. On a similar basis, some hypothyroid states have been considered to depend on a reduced secretion of TSH.

Definite evidence bearing on these suppositions depended on determinations of the amount of TSH secreted by the pituitary gland or circulating in the body fluids in normal and pathological conditions. Unfortunately, methods available until recently were not sufficiently sensitive to detect the presence of circulating TSH in normal individuals nor to show small variations in pathological conditions. Hence the results reported in the literature are either negative or contradictory.

A new and much more sensitive method for the determination and assay of TSH was described in 1944 by De Robertis and Del Conte (2). This method is based on determinations of the number of colloid droplets found in cells of the guinea pig thyroid prepared by freezing-drying, following injection of an extract of blood containing TSH. This cytological method is several hundred times as sensitive as previous methods, and is capable of detecting the injection of 0.0002 Junkman-Schoeller (JS) unit of TSH. It was found that the number of colloid droplets was nearly a linear function of the logarithm of the dose of TSH. The response curve could be used for making approximate assays of the TSH injected (Fig. 1).

This paper deals with the assay of TSH in the blood of man and presents results obtained in 13 patients with thyroid disease and in 3 normal controls. The study had to be terminated before a statistically significant series could be accumulated. The number of cases is hence too small to permit

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* Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts. This work was mainly done at the Institute of General Anatomy, University of Buenos Aires, Argentina, in 1944–46.

definitive analysis of all our findings. Our data are presented in order to call attention to the value of this method as an investigative tool, to report the assays for TSH, and to comment on the possible physiological and clinical significance of our findings. From these data it appears that there may be a definite range of concentration of TSH in the circulating blood

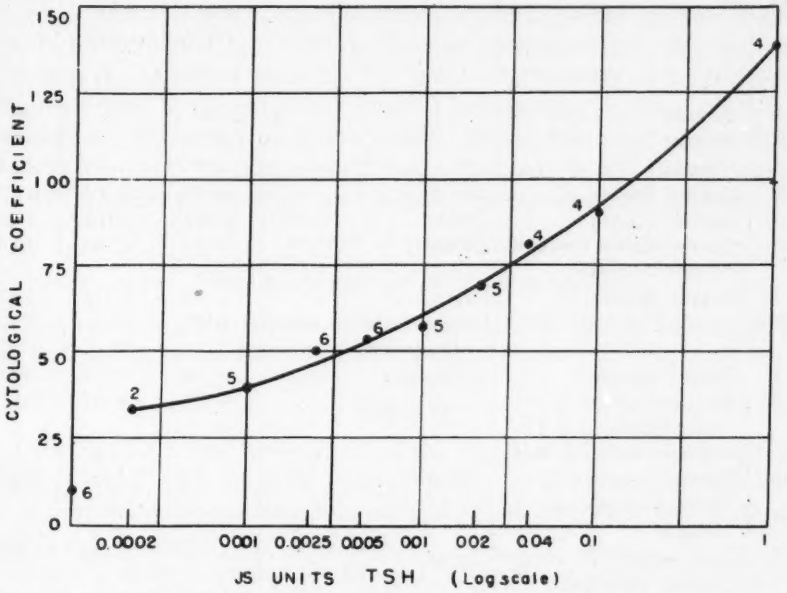

FIG. 1. Curve indicating cytological coefficients of the thyroid gland of guinea pigs as a function of the amount of injected thyrotropic hormone (TSH) in Junkman-Schoeller (JS) units. Numerals along the curve indicate the number of guinea pigs used in each determination (De Robertis and Del Conte (2)).

of normal individuals, and that the concentration is diminished in certain pathological states and increased in others, sometimes considerably.

METHOD

Ten to 20 ml. of blood[1] was taken by venopuncture and treated by Fellinger's method (3). The blood was hemolyzed by adding distilled water up to a total volume of 50 ml.; 45 ml. of acetone was added, and the mixture filtered after standing a few hours in the refrigerator. Acetone was then added to the filtrate in an amount sufficient to make the total acetone con-

[1] The author is greatly indebted to Prof. E. del Castillo and Dr. A. Rey for providing most of the material and clinical data presented in this paper.

TABLE 1. CLINICAL DATA, TREATMENT AND CYTOLOGICAL COEFFICIENTS (C_e) OF PATIENTS REPORTED IN THIS PAPER

Case No.	Diagnosis	Treatment	Initial basal metabolic rate %	Basal metabolic rate at time of TSH assay %	C_e
1	Normal				39
2	Normal				47.8
3	Normal				48.2
4	Graves' disease	None	+ 65	+ 65	11.8
5	Graves' disease	None	+103	+103	16.5
6	Graves' disease (residual hyperthyroidism)	None	+ 55	+ 55	16.9
7a	Graves' disease	None	+ 75	+ 75	14.0
7b		Assay 48 hours after thyroidectomy	+ 75		39.2
8	Graves' disease	Thiouracil	+ 80	+ 2	40.7
9	Recurrent toxic goiter, exophthalmos and localized edema of legs	None	+ 47	+ 47	115.7
10	Graves' disease with edema of legs and hands	None	+ 57	+ 57	101.6
11	Toxic goiter with severe ocular proptosis	None	+ 54	+ 54	135.0
12a	Toxic goiter ("ophthalmic type")	None	+ 55	+ 55	101.9
12b		Desiccated thyroid	+ 55		47.2
13	Spontaneous myxedema	After 1 week of treatment with desiccated thyroid	− 34		73.6
14	Postradiotherapeutic myxedema	None	− 21	− 21	156.2
15	Cretinism	Intensely treated with desiccated thyroid	− 19	+ 38	15.3
16	Pituitary myxedema	None	− 14	− 14	19.8

tent 85 per cent. The precipitate was separated by centrifugation, dried in vacuo at 40° C., and redissolved in distilled water (pH of solution about 6.0). After standing a few hours the solution was either injected directly or after short centrifugation to remove undissolved particles. Guinea pigs weighing 150–300 Gm. were lightly anesthetized with ether and an amount of fluid equivalent to 2 ml. of blood was injected into the heart. The ani-

mals were sacrificed by a blow on the head thirty minutes after the injection, and the thyroids were dissected out as quickly as possible and frozen in isopentane at minus 195° C. or in liquid air. The best cytological results were obtained by putting the gland between two pieces of muscle prior to the freezing. The glands were then dried *in vacuo* at minus 30° C. in Gersh's apparatus, treated in absolute alcohol to denature the proteins and embedded in nitrocellulose. Sections were stained as previously described by De Robertis in 1941 (4). TSH was assayed cytologically by the method of De Robertis and Del Conte (2). The number of droplets in ten follicles of the inner zone of the gland was counted and the total number divided by the sum of the average diameters of the follicles. This quotient multiplied by 100 represents the number of droplets contained in a hypothetical follicle 100 microns in diameter, and is called the cytological coefficient (C_c).

$$C_c = \frac{\text{number of droplets} \times 100}{\text{sum of the average follicular diameters}}$$

The results so obtained[2] were correlated with the clinical history, physical findings and laboratory data (Table 1).

RESULTS

Controls. TSH was determined in the blood of 2 normal adult males (cases 1 and 2) and 1 normal adult female (case 3). C_c ranged from 39 to 48.2, corresponding to concentrations of blood TSH between 0.005 and 0.0025 J-S unit per ml. (Figs. 1 and 2).

Figure 3 shows a guinea pig thyroid before the injection and Figure 4 shows it after the injection of the animal with 2 ml. of blood from a normal individual.

Hyperthyroidism with low TSH. This category includes 4 untreated patients with hyperthyroidism (cases 4, 5, 6, 7a) and two treated patients (cases 7b, 8) without predominant ocular signs, but not otherwise differing from typical Graves' disease, except as noted below. Case 6 was an instance of recurrent hyperthyroidism following a partial thyroidectomy a few months before.

The C_c in these 4 untreated patients ranged from 11.8 to 16.9. As the C_c of the normal guinea pig is 9.4, these assay values indicated that the circulating TSH in the blood of these patients approached zero (Figs. 1 and 2). Case 7a was a patient treated by thyroidectomy, after which (7b) the C_c rose to 39.2 (Fig. 2). In case 8, the patient showed a C_c of 40.7 after treatment with thiouracil (Table 1). Both of these values appear to be within normal limits.

[2] Thanks are given to Dr. E. Del Conte and Dr. A. Villamil for technical assistance.

Hyperthyroidism with high TSH. In this group fall 4 cases classified as toxic goiters. These 4 untreated patients showed some special symptoms in addition to those of hyperthyroidism (Table 1).

Case 9 was a patient who showed exophthalmos with persistence of toxic symptoms even after two operations (May 1940, May 1941), localized edema of the legs, cyanosis, hardening and dryness of the skin and edema

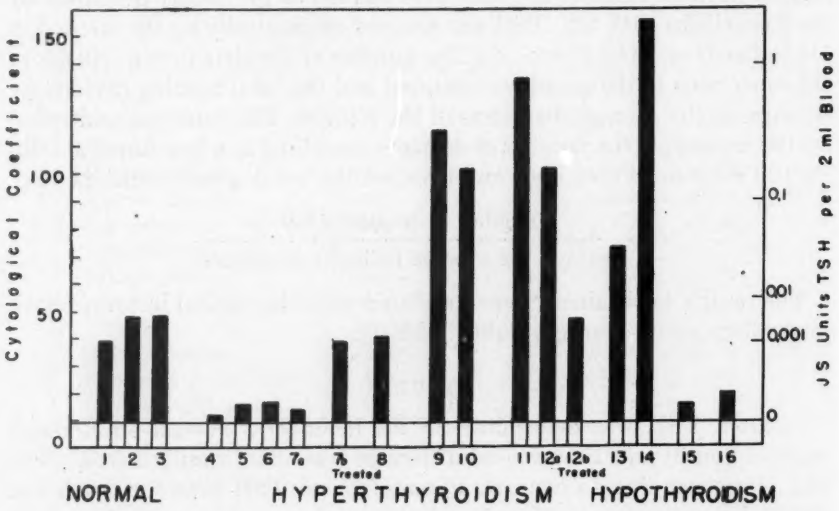

Fig. 2. Diagram indicating cytological coefficients (left ordinate) and corresponding concentrations of thyrotropic hormone in the blood (right ordinate) of the 16 men and women studied. For detailed indications see Table 1.

of the face. The patient in case 10 had a diffuse toxic goiter and edema of the legs and hands. Patient 11 showed exophthalmos with severe proptosis. Case 12a was a patient, as in case 11, with hyperthyroidism of the "ophthalmic type" (Hertz, Means and Williams (5)). All these patients showed

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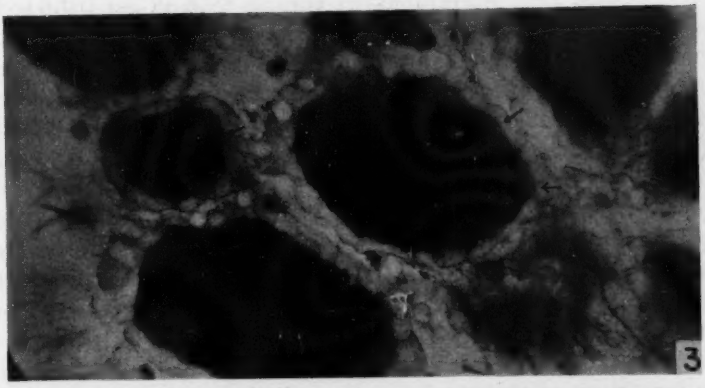
Explanation of Plate 1

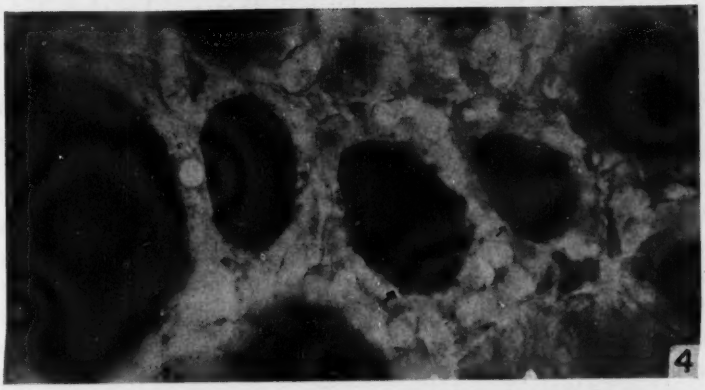
Fig. 3. Thyroid gland of an untreated control guinea pig. The epithelium shows only a few colloid droplets.

Fig. 4. Thyroid gland after guinea pig was injected with extract of 2 ml. of blood of a normal human subject (case 2). Note increase in colloid droplets in cells. Some droplets are being excreted into the lumen.

Fig. 5. Thyroid gland after guinea pig was injected with extract of 2 ml. of blood from a patient with toxic goiter and severe ocular proptosis (case 11). Note the considerable increase of colloid droplets in cells, and the higher epithelium.

Guinea pig thyroid glands frozen, dried and stained with anilin blue-orange.





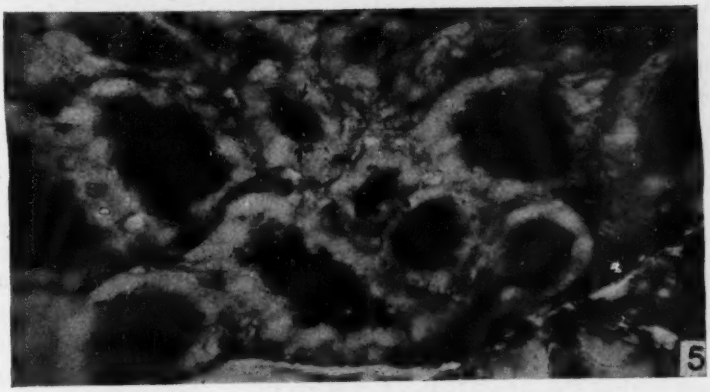


PLATE 1

evidence of blood TSH levels far above normal, with a C_e of 101.6 to 135.0, corresponding to TSH concentrations over 0.05 JS unit per ml. of blood.

Figure 5 shows the increase in colloid droplets induced by the blood extract from the patient in case 11 (see Fig. 2). In an additional observation the patient in case 12a was treated for several weeks with desiccated thyroid (case 12b), whereupon the C_e diminished considerably and reached the normal range (47.2).

Hypothyroidism with high TSH. Two cases fall in this classification. The patient in case 13 was 27 years of age and showed typical spontaneous myxedema of ten years' standing. The basal metabolic rate prior to treatment was minus 34 per cent. TSH assay, performed one week after starting treatment with desiccated thyroid, showed a C_e of 73.6.

The patient in case 14 was 28 years of age and presented myxedema following treatment of thyrotoxicosis by radiotherapy. Exophthalmos was persistent, although the basal metabolic rate was minus 28 per cent. No treatment was started prior to TSH assay. The C_e of 156.2, corresponding to over 0.5 JS-TSH unit per ml., was nearly 100 times the normal value.

Hypothyroidism with low TSH. In case 15 the diagnosis was cretinism. The patient was 28 years old and had been treated briefly with desiccated thyroid at the age of 10 and again at the age of 20. The initial basal metabolic rate was minus 19 per cent. TSH assay, performed after two months of intensive thyroid therapy, at which time the BMR was plus 38 per cent, showed a C_e of 15.3.

The patient in case 16, 42 years of age, had an operation and radiotherapy for a pituitary tumor at the age of 19. Progressive myxedema and hypogonadism developed between the ages of 25 and 30. Desiccated thyroid had been administered at intervals during this time, with definite temporary improvement. TSH assay performed after a long interval without treatment, at which time the basal metabolic rate was minus 14 per cent, showed a C_e of 19.8 (Fig. 2).

Assays in cases 15 and 16 indicated a concentration of blood TSH approaching zero.

DISCUSSION

The sensitivity of the cytological method. The present method is the first to be reported which is sufficiently sensitive to detect definitely and to measure the level of circulating TSH in normal blood. The success of the procedure depends on separation and extraction according to Fellinger's method, which removes the fraction of the blood containing the thyroid hormone so that almost no iodine is left (Fellinger (3); Collard *et al.* (6)), and permits the recovery of TSH after it has been added to the blood (Cope (7); Robinson (8)). The injection of whole blood or serum produces no

detectable colloid-droplet response in the thyroid. This may indicate that TSH action may be antagonized by other substances in blood, perhaps by the thyroid hormone.

Previous methods were not sufficiently sensitive to detect reliably and consistently the small concentration of TSH in normal blood. Although Fellinger (3) claimed to have detected TSH in 10 ml. of normal blood when injected into the guinea pig, Cope (7) was unable to find any effect even when extracts of 50 ml. were administered, and Collard *et al.* (6) also reported negative results in attempts to find TSH in the blood of 12 normal individuals.

Rawson and Salter (9) state that 0.125 JS unit of TSH is the smallest amount detectable with the microhistometric method based on the chick. The present method, based on the guinea pig, permits the detection of 0.0002 JS unit.

A comparison of species shows the guinea pig to be the most sensitive of the animals which have been tried in the colloid-droplet cytological assay method. The hypophysectomized rat is completely insensitive even to the administration of 0.01 or 0.1 JS unit (De Robertis and Del Conte, unpublished results), although the normal rat does respond to such doses (Grasso (10, 11)). Newly hatched chicks have been tried with this method by Dvoskin (12) who fixed the thyroids in Carnoy's fluid and was able to detect a minimum amount of about 0.005 JS unit. His data show a wide scatter, which may reflect his fixation technique, his method of injection (subcutaneous), or the peculiar character of the colloid droplets in the thyroid of the fowl. Junqueira ((13) and personal communication) states he has run into difficulty on this score with the chick thyroids, even when the freezing-drying method was used, because of the small size of many colloid droplets.

The normal level of circulating TSH. Our 3 normal cases present too small a series to permit final conclusions as to the range of circulating TSH which can be regarded as normal. However the assays yielded values sufficiently close to suggest provisionally that the normal TSH level in human blood corresponds to a C_e of 39–49, corresponding to 0.005 to 0.0025 JS unit per ml. It is hoped that more definitive values may soon be established, based on a statistically significant series of cases.

TSH values in hyperthyroidism. The 8 patients cited here fall into two groups: 4 with typical Graves' disease, that presented a low TSH level; and 4 which had, in addition, other symptoms such as edema and severe exophthalmos and showed greatly elevated TSH levels. These findings suggest that in hyperthyroidism in man there may be at least two clinical syndromes, one characterized by excess pituitary production of TSH and the other characterized by low TSH synthesis, perhaps due to the inhibitory effect of

the excess circulating thyroid hormone. The data do not support the idea that "classical" Graves' disease may be due to excessive secretion of TSH, but suggest that the "ophthalmic type" and other atypical Graves' syndromes may depend on this mechanism.

If this tentative differentiation of hyperthyroidism into two types can be sustained by subsequent systematic investigations, it might provide a therapeutic rationale by which therapy could be directed at the organ primarily responsible for the disease in each case (Thompson and Thompson (14)).

Previous efforts to determine the level of TSH in the blood in hyperthyroidism (6) or of TSH in the urine (3, 7, 8, 15, 16, 17, 18, 19) have been also conflicting or equivocal.

Effect of treatment of hyperthyroidism. The fact that in all 3 of our treated patients with hyperthyroidism (cases 7b, 8, 12b) the TSH reverted to apparently normal levels as their clinical condition improved, suggests that the treatment helped to restore more normal thyroid-pituitary relationships.

TSH values in hypothyroidism. This series of 4 cases, is too small and variable to permit rigorous interpretation. It does suggest, however, that there also may be two types of hypothyroidism, with low and with high TSH respectively, representing cases in which hyposecretion of TSH by the hypophysis may be the primary cause (case 16), and others in which the primary deficiency is in the thyroid (cases 13 and 14). It would appear further that assays of circulating TSH might be of value in differentiating these two types, and as therapeutic guides.

Previous workers have reported finding TSH in the blood of hypothyroid patients (6) and in the urine (17, 18, 20, 21, 22).

SUMMARY

A very sensitive cytological method has been applied to the assay of thyroid stimulating hormone (TSH) in the blood of man.

In three normal individuals the level of TSH in the blood ranged between 0.005 and 0.0025 Junkman-Schoeller unit (JS) per ml.

Four patients with hyperthyroidism of the "classical" Graves' disease type showed very small amounts of circulating TSH, the values approaching zero. The concentration rose in one case to a normal level after thyroidectomy and was also found to be normal in another case after treatment with thiouracil.

Four other patients with hyperthyroidism showed very high TSH levels in the blood. Clinically two of these patients were of the "ophthalmic type"; one had permanent ocular signs and localized edema of the legs, and the

other, edema of the legs and hands. In one of the ophthalmic cases, treatment with desiccated thyroid lowered the TSH level to normal.

Two patients with hypothyroidism had high levels of TSH, whereas in two others the concentration was very low, approaching zero. One of the latter also had pituitary deficiency.

These results are discussed from the point of view of their possible physiological and pathogenetic implications.

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THE URETHRAL SMEAR IN THE NORMAL HUMAN MALE

MILDRED T. VOGEL, M.S., THOMAS H. McGAVACK, M.D. AND HENRY KAMMANDEL, M.D.

From the New York Medical College, Metropolitan Hospital Research Unit, Welfare Island, New York, N. Y.

CHANGES in the vaginal secretion which can be correlated with the menstrual cycle have been recognized since 1933 (1). The vaginal smear technique has been applied to the diagnosis of functional disturbances originating in the female endocrine system and to the evaluation of therapeutic measures used therefor. More recently attention has been focussed on its use in the diagnosis of malignant diseases of the female genital tract (2). Similar techniques have also been employed in the detection of malignant disease of the respiratory, genito-urinary and gastro-intestinal tracts of both men and women. (3).

In a recent article (4) a clinician reported that the cells observed in the smears taken from the urethras of men, in whom sex hormone deficiency existed, differed from those observed in so-called "normal" subjects. Moreover, the same worker stated that, following administration of androgen sufficient to relieve symptoms, the pattern of the urethral smear returned to normal. On the basis of his preliminary studies he has suggested the use of urethral smears in the male as a test for hormone deficiency and has submitted his findings in order that others may try the method and "prove or disprove its accuracy and value." In accordance with this suggestion another investigator (5) has published data which he believes invalidate the diagnostic significance of the urethral smear on the grounds that there are no significant differences in the smears of normal and hypogonadal males, either before or after treatment.

Before attempting to use the urethral smear as an index of sex hormone deficiency or as an aid in evaluating therapy it seemed imperative to us that a study be made of the smears of normal males in different age groups over a period of time with a view to ascertaining answers to the following questions:

1. What cell types are present in the urethral smear and from what part of the genito-urinary tract does each arise?
2. What are the relative percentages of different types of cells and do they differ in the various age groups?
3. What are the staining properties of the cells? Do these properties

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TABLE 1. OBSERVATION OF URETHRAL SMEARS OF 23 NORMAL MALES

Case	Age Yrs.	Total No. smears taken	Total No. smears containing adequate data	Cell types expressed in approximate per cent (to nearest 5) and degree of leucocytosis													
				Cornified cells		Superficial cells, acidophilic		Superficial cells, basophilic		Intermed iate cels, acidophilic		Intermediate cells, basophilic		Round cells, acidophilic & basophilic		Leucocytes	
				Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	
J.V.	18	81	40	5–40	20	5–20	10	20–65	45	0–10	5	10–30	20	0–10	0	0–+	
C.K.	18	72	42	5–55	25	0–35	15	10–65	25	0–15	5	10–45	25	0–45	5	0–++++	
L.B.	19	39	29	10–50	25	5–20	10	10–40	25	0–15	5	0–50	30	0–20	5	0–++++	
J.B.	25	82	64	10–60	30	5–20	10	5–35	20	0–20	10	5–35	20	0–30	10	0–++++	
S.S.	31	109	58	5–80	50	0–25	15	0–50	15	0–20	5	0–40	10	0–40	5	0–+++	
C.Y.	22	78	60	5–60	30	0–20	10	0–30	10	0–35	15	0–35	10	5–50	25	0–++++	
R.R.	19	84	63	0–50	20	0–25	10	0–55	20	0–25	5	5–45	20	0–55	25	0–++++	
J.M.	32	122	94	5–60	25	0–35	15	0–65	10	0–25	15	0–40	10	0–70	25	0–++++	
F.M.	18	80	68	5–85	45	0–25	10	0–80	30	0–15	5	0–40	10	0–20	0	0–+++	
H.K.	30	100	77	25–75	55	5–35	20	5–30	10	0–20	5	0–25	5	0–20	5	0–++++	
H.Ke	19	81	74	5–50	25	0–20	5	5–30	10	0–15	5	0–40	20	5–65	35	0–++++	
R.E.	28	7	7		20		10		10		15		20		25	++++	
J.Bi	52	5	5	0– 5	0	5–10	5	40–65	50	0	0	25–45	35	0– 5	0	0	
G.D.	50	40	19	0–60	40	0–25	20	10–30	20	0–15	10	0–35	5	0–25	5	0–+	
J.Ma	44	3	3	10–20	15	5–20	10	20–60	40	0–20	10	20–25	20	0– 5	5	0	
J.L.	58	3	3	0–10	5	0–15	5	50–65	60	0	0	20–35	25	0– 5	5	0	
M.O.	56	3	3	10–30	15	5–30	15	35–65	50	0– 5	0	5–30	20	0	0	0	
J.J.	53	50	45	0–40	10	0–25	5	15–75	35	0–25	10	0–55	30	0–35	10	0–++	
J.Fa	11	4	4	0–10	5	5–25	20	45–70	50	0	0	0–40	25	0	0	0	
R.Ro	9	3	3	15–50	30	10–20	15	10–30	20	5	5	15–35	25	0–15	5	Occ.	
J.Mu	9	3	3	0– 5	5	5–10	5	45	45	0	0	40–50	45	0	0	0	
R.S.	11	3	3	10–15	10	10–15	10	40–45	40	0– 5	5	30–35	35	0	0	0	
C.S.	11	4	4	25–75	35	10–20	10	10–45	25	0–10	10	0–25	15	0–20	5	Occ.	

vary from individual to individual? Do they vary from day to day in the same subject?

4. Are any cyclic variations demonstrable?

The standard texts give almost no detailed information on the histology of the distal portion of the male urethra. Hinman (6) states that the central portion of the urethra is lined by stratified columnar epithelium and that this becomes squamous from the outer end of the fossa navicularis, merging with similar squamous epithelium at the meatus and glans penis. Maximow (7) states that the pars cavernosa, or central portion of the urethra, is lined by stratified or pseudostratified columnar epithelium and that patches of squamous epithelium are common in the cavernous parts. He also states that squamous epithelium occurs as a rule in the fossa navicularis. The exact sites of transition of one type of epithelium to another and variations in the number of cell layers in the mucosa of the distal urethra, meatus and glans penis of males at various ages becomes a problem worthy of investigation. It is unfortunately dependent on access to a rather large volume of autopsy material.

MATERIALS AND METHODS

A. *Subjects.*

Twelve men, ranging in age from 18 to 32 years, in normal health, volunteered to take urethral smears daily for periods of time which varied from 7 to 122 days; 6 men, 44 to 58 years of age, procured smears for shorter periods of time, 3 to 50 days; and smears were obtained every other day for a one-week period from 5 boys ranging in age from 9 to 11 years (Table 1).

B. *Technique of gathering material and its preparation for study.*

In all but the group of very young boys the smear was taken immediately upon arising each morning. This involved holding the lips of the meatus open and pressing a clean, dry slide against the mucosal surface. This method was routinely adopted after efforts had been made to secure material by suction with a number 8 catheter attached to a syringe, by cotton swabs and by insertion of a small glass rod for a depth of a few millimeters. The slide was immediately immersed in fixing solution (equal parts of ether and 95 per cent alcohol). The slides were prepared for study in the same manner as vaginal smears; the PGI stain (8) was employed throughout. Tabulation of observations was made daily and included an approximation of the percentage of each type of cell present, its staining properties, the absence or presence of leucocytes, the degree of leucocytosis (expressed as 0 to $+++$, Tables 1 and 2), and the presence of material such as mucus, bacteria and debris. The percentages of types of cells were

estimated only after all of the smear was inspected because, in many instances, great variations in the contents of different fields in the same slide were noted. In the present investigation a total of 1056 slides have been examined. About 25 per cent of the total number of smears did not contain sufficient material for critical study; they are consequently omitted from the tables.

TABLE 2. PER CENT OF CELL TYPES OBSERVED IN URETHRAL SMEARS—AVERAGES FOR THREE AGE GROUPS

No. of subjects	Average age Yrs.	Total No. smears examined	Total No. smears reported	Cornified cells	Superficial cells		Intermediate cells		Round cells	Leucocytes	
					A*	B*	A*	B*	A* & B*		
				Range Av.	Range Av.	Range Av.	Range Av.	Range Av.	Range Av.	Range	Av.
Group I 12	23	935	676	0–85 30	0–35 10	0–80 20	0–35 10	0–50 15	0–70 15	0–+ to 0–++++	0–++++
Group II 6	52	104	78	0–60 15	0–30 10	10–75 45	0–25 5	0–55 25	0–35 5	0 to 0–++	0
Group III 5	10	17	17	0–70 15	5–25 10	10–70 35	0–10 5	0–50 30	0–20 5	0 to occ.	0

* A—acidophilic.
* B—basophilic.

As the work progressed it seemed advisable to gather additional data which might have had some bearing on our interpretation of the smears. Therefore, for a period of approximately six weeks, 9 of the subjects in Group 1 kept daily records of the pH of the meatus at the time the smear was made, and of the last specimen of urine voided prior to taking the smear.

Individual graphs were set up for each subject to indicate the approximate percentage of the different cell types and pH of meatus and urine in order to visualize better the daily alterations in their smears and for the purposes of more readily comparing the data from one individual with those of another (Fig. 1).

C. *Cell nomenclature.*

Four types of epithelial cells have been observed in the smears. These have been somewhat arbitrarily named: I. The large, wafer-like squamous cell, with either a small pyknotic nucleus or a "ghost nucleus," which takes an orange stain, is identified as a *cornified* cell. II. A cell, similar in size and shape, with either a pyknotic or relatively small, vesicular nucleus, is called a *superficial* cell. Its cytoplasm takes either a grayish-blue or bluish-green stain, or a pink to pinkish-orange stain, indicating either basophilic or acidophilic properities, respectively. The nucleus invariably takes a blue to violet stain. III. A smaller cell with similar staining properties,

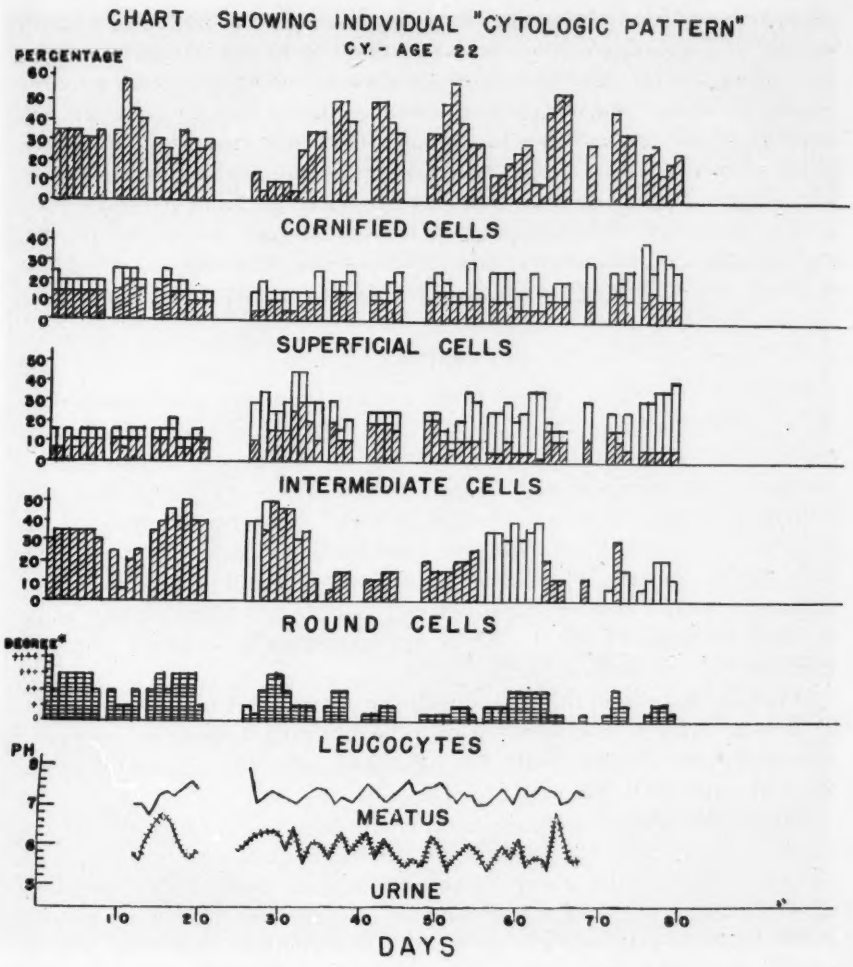

FIG. 1

having less irregularity of outline and an ovoid or a somewhat elongated form, is termed an *intermediate* cell. The nucleus, also ovoid, is larger in proportion to the total cell size than in the superficial cell. It exhibits a fine patterning of the chromatin and stains a blue or violet color. IV. This

cell type is considerably smaller; it is approximately one-half to two-thirds the size of the intermediate cell and tends to be round or slightly oval in shape. The nucleus, of similar shape, occupies about one-half or more of the volume of the cell: the nuclear chromatin assumes a fine, lacy pattern, but in many of our preparations this is not too clearly visualized. The cytoplasm of this cell also exhibits both basophilic and acidophilic properties. The nucleus may vary from deep blue to violet or from red to reddish brown, apparently independently of the cytoplasmic variations, for not infrequently a reddish-brown nucleus was observed in cells the cytoplasm of which was definitely basophilic. We have designated this the *round* cell (Tables 1 and 2).

RESULTS

Group 1: The major portion of the smears examined was furnished by the 12 subjects comprising this group. Although all four types of cells were not always present on a single smear they were observed at one time or another in the smears of each subject studied. There is evidence that each individual tends to have a "cytologic pattern." However, the range of the percentages of cell types noted over the total period of observation for each subject is very wide. A more accurate concept of the individual cytologic pattern and its variations can be obtained from a study of individual averages (Table 1). There appears to be no evidence that the variations are cyclic in nature.

When the data from these 12 subjects are averaged it will be seen (Table 2) that the types of cells occurred with the following frequencies, expressed in percentages: cornified cells, 30; superficial cells, 30; intermediate cells, 25; and round cells, 15.

Most striking among observations of the cells, per se, were the changes in the staining properties of superficial, intermediate and round cells. At frequent intervals the smears of any one subject would show a decidedly altered ratio of acidophilic to basophilic cells. For example, the smears of F.M. consisted principally of cornified and acidophilic superficial cells for several days and then changed to predominantly basophilic superficial cells and/or to a smear in which all cell types were present (Figs. 2, 3 and 4).

Although the cells themselves were usually well delineated, almost all smears contained debris which we believe is cellular in origin. This conclusion was reached because, in numerous instances the transition from nucleated cell to anuclear form and then to small fragment appeared to be well portrayed. The debris tended to take either the acid or basic stain of the cells in its immediate vicinity.

Other urethral contents which were observed in all subjects, although

FIG. 2. F.M. 4-14-47—Cornified cells. Note "ghost" nuclei and refractile body. ×200.

not present in every smear, were polymorphonuclear leucocytes, mucus, bacteria and elongated, refractile bodies. The leucocytes were observed particularly in those smears which contained round cells, and vice versa (Fig. 5). Indeed, it deserves emphasis that their numbers were roughly and directly proportional to one another. Mucus, which appeared as a fine granular deposit, or background "shading," stained a faint lavender or blu-

FIG. 3. F.M. 2-17-47—Superficial cells. These cells are basophilic. ×200

ish color. It was present in inconspicuous amount in the majority of smears examined. Small bacillary forms of bacteria were noted in a few smears. Cocci were not present in any of the smears of this, or of the other two groups. Occasionally, smears from men of Groups 1 and 2 contained numerous spermatozoa. The refractile bodies mentioned above were seen from time to time in smears from Groups 1 and 2. These were filamentous in character, and varied in length from a few micra to sizes exceeding the limits of a single high-power field. Straight, or more frequently curling like a loose string, they either failed to take the stain or appeared as pale

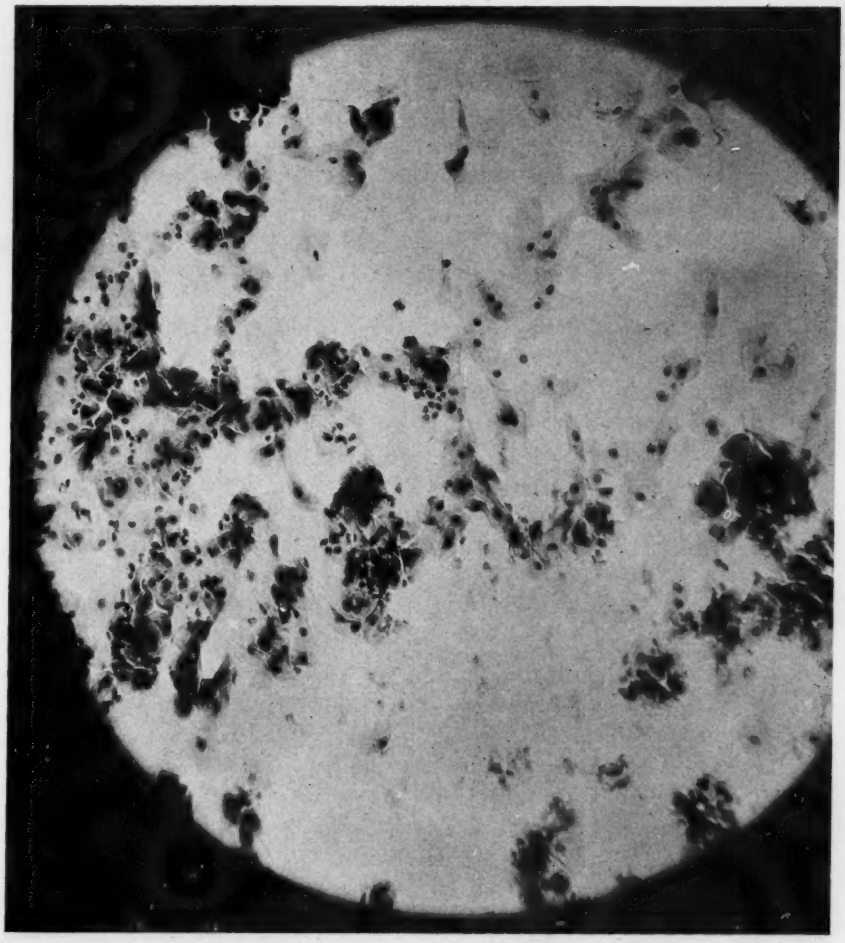

FIG. 4. F.M. 3-15-47—Epithelial cells of all types and leucocytes are present in this smear. ×200.

yellowish-green to pale brown objects. Very often clumps of cells extended down the length of one or both sides, as if glued there, in haphazard fashion (Fig. 2).

The pH of the meatus for group 1 ranged from 5.0 to 7.5, with an average of 6.7. The urinary pH varied from 4.5 to 7.5, with an average of 5.8. Day-to-day changes in pH of meatus and urine could not be correlated with the day-to-day changes in percentages of cell types observed in the smears nor with their staining properties (Fig. 1).

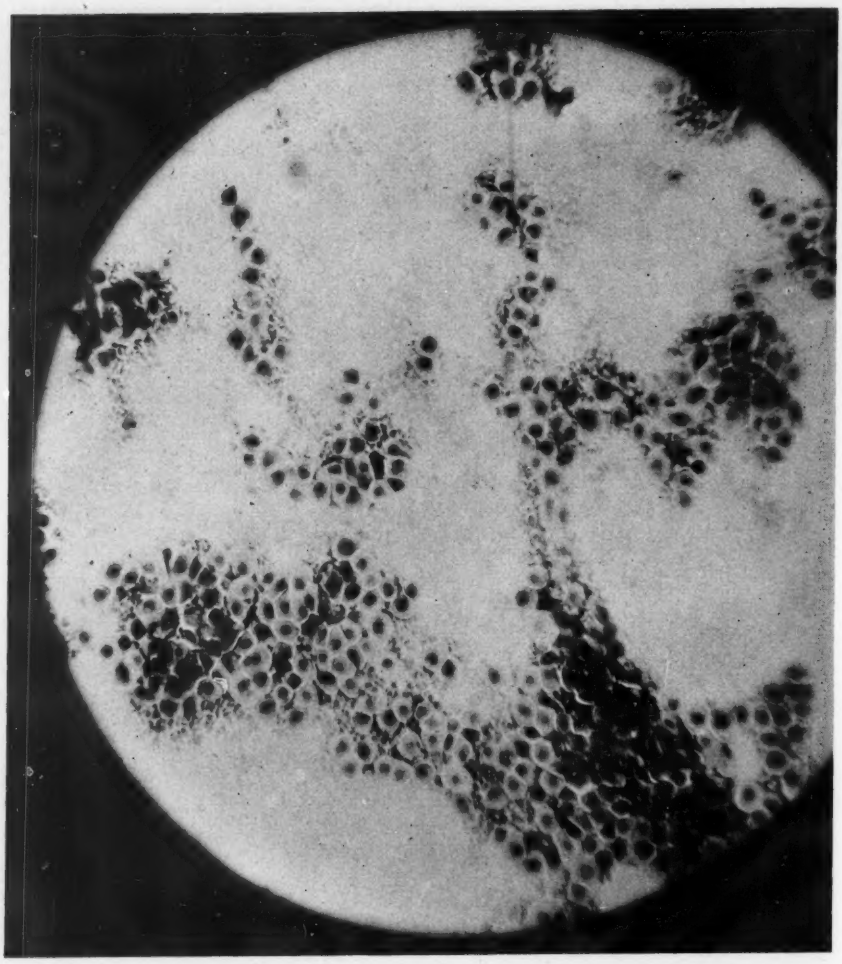

FIG. 5. J.M.—Smear containing round cells. Note presence of leucocytes. ×200.

Group 2: In this oldest age group investigated, individual cytologic patterns occurred, similar to those noted for the men of Group 1 (Tables 1 and 2). The study of the averaged percentage of cell types, however, shows some differences. Superficial and intermediate cells, preponderantly basophilic, comprised 55 and 30 per cent, respectively, of all cell types present. Cornified cells, although present in relatively high numbers in the smears of one man aged 50 (G. D.), averaged approximately only 15 per cent of the total number of cells. Round cells were fairly prominent in several of the smears of 2 subjects (G. D. and J. J.) but were infrequently

seen in the majority of smears examined and averaged approximately 5 per cent of the total cellular material. Concomitantly, a lesser degree of leucocytosis existed for this group: polymorphonuclear leucocytes were observed in the smears of only 2 subjects (Tables 1 and 2). Debris was present to a greater or lesser degree in all smears. Mucus, bacillary forms and refractile bodies, as previously described, were observed from time to time.

Group 3: Seventeen smears were collected from 5 boys, ranging from 9 to 11 years in age. Superficial and intermediate cells, averaging approximately 45 and 35 per cent respectively, of the total, took the basic stain more frequently in this, as in the older age group. Cornification was moderately prominent in the smears of 2 boys (R. Ro. and C. S.) but, on the average, cells of this type comprised only 15 per cent of the total. Round cells and occasional leucocytes were observed only in the smears of the 2 boys who also exhibited relatively higher numbers of cornified cells (Tables 1 and 2). Debris was present in lesser degree than in the smears of the other two groups and mucus, bacteria and refractile bodies were not noted in these smears.

DISCUSSION

A. *Cellular and other constituents of the urethral smear.*

The various characteristics of the cells present in the layers of squamous epithelium of the skin and some bodily orifices need no reviewing. We may accept unquestionably the presence of superficial and intermediate squamous cells, in the normal urethral smear. The exact site of origin of the round cell is somewhat obscure. Desquamation of basal cells of the lower layers of squamous epithelium has been noted in samples of the vaginal secretions obtained from women in whom atrophic changes were apparent. Due to the paucity of material taken from the distal urethra and glans penis for microscopic study, mention of the number of layers of cells comprising the epithelium of this region has not been found in a rather extensive, although admittedly not complete review of the literature. If there be but few layers present one can more readily postulate that the round cell observed in these smears originates in the lower epithelial layers: at the same time one might want to correlate this with a rapid growth, maturation and desquamation process of the entire epithelial surface in contrast with that seen in the female genital tract, in which, normally, only cells of the upper and middle layers are shed. Until more information is available the possibility should not be overlooked that the round cell represents a form which is transitional between the columnar cells of the cavernous part of the urethra and the squamous cells of the outer part of the fossa navicularis and meatus.

It is not known whether the debris, a constant feature of the smears, represents local material or material originating throughout the length of the urethra. Its amount, relatively high when compared with the total amount of material collected, may lend support to the validity of the theory of rapid regeneration of epithelium in this anatomic region.

The positive correlation of round cells and leucocytes suggests that the former cells originate in proximity to the lamina propria where interstitial fluid is apt to contain numerous phagocytes.

Mucus is secreted by the columnar cells lining the glands of Littre, which are situated throughout the length of the urethra, and particularly on the dorsal surface. Mucus was present in varying degree in the smears taken by the subjects of Groups 1 and 2. Its absence from the smears of the youngest males may indicate a prepubertal lack of development of these glands.

The bacillary forms of bacteria observed in the smears are thought to be smegma bacilli and nonpathogenic micro-organisms.

Although the nature and origin of the refractile bodies described is unknown, it is believed that they may emanate from the accessory sex glands, such as the prostate, or from intra-epithelial cysts, which Maximow (7) states are of frequent occurrence in the cavernous urethra. These cysts contain a colloid-like substance.

B. *Factors which may influence the character of the urethral smear.*

1. *Mechanical:* In attempting to explain the variations observed from time to time in the constituents of urethral smears of the individual or groups of individuals, several factors must be taken into consideration. Hinman (6) states that the meatus varies in size, position and appearance with the nature of the embryologic closure of the urethral groove. In separating the lips of the meatus, therefore, the amount of exposed surface area should vary from individual to individual. We have found this to be true, for, in testing several subjects prior to starting this investigation we encountered a number who proved unsuitable for study because of the small diameter of the urethral orifice. The pressure exerted by the individual in obtaining the smear is a second factor which may influence the number and nature of the elements present.

With the exception of the mucus, and possibly the refractile bodies, we believe that the greater part of the material present in the fossa navicularis and on the inner surfaces of the meatus is local in origin. In many men the the valve of Guerin, situated at the proximal end of the fossa navicularis, is narrower than the external meatus. This may serve to retain those urethral contents which collect during periods between micturition.

2. *Chemical or metabolic:* Variations in the staining properties of the different cell types are not too readily explained. Experimentation with different staining procedures was not attempted and it is possible that some other technique would be better adapted to this material and would constitute a more reliable index of basophilism and acidophilism.

No demonstrable relationship existed between the pH of the meatus and/or urine and the variations in the staining properties of the cells.

The probability must be entertained that a metabolic or hormonal basis for variations in staining exists. In women, the amount of follicular hormone secreted can be directly correlated with the number of cornified and acidophilic cells present in the vaginal secretion. However, the presence of these same cell types, in far lesser numbers, has been noted in smears from castrates of different age groups and from postmenopausal women. In this connection we have expressed the belief that tissue growth and maintenance is under the control of the endocrine system as a whole (9). Such a conclusion would be equally applicable here: the degree of cornification and acidophilism may be dependent on fluctuations in the hormonal balance, particularly as it concerns the pituitary, adrenal and gonad. It is of interest to point out in this connection that values for the total urinary excretion of 17-ketosteroids vary and there is "some evidence that ketosteroid output decreases with increasing age" (10). In our laboratory maximum values have been noted in those males who are between the ages of 20 and 40 years. Cornification and acidophilism of cells was more pronounced in our subjects of like age and, in particular, in those men who were closest to 30 years of age.

C. *The urethral smear as a diagnostic aid.*

Before discarding or accepting the urethral smear as an index of deficiency of sex hormone in the male or as an objective aid in determining the effects of therapy certain points should be elucidated. The optimum stain for urethral material must be sought. The nature of the change in staining reaction needs further study. The relative percentages of the different types of cells vary from age group to age group to a moderate degree. Therefore, the greatest difficulty currently apparent is to describe a smear which can be accepted as characteristic of any given functional state, such as prepuberty, puberty, active sexual life, and the climacteric. For instance, in treating the male climacteric it would be helpful to recognize certain characteristics in the urethral smear which could serve as an objective gauge for the adequacy of therapy. In order to determine whether or not this can be done it will be necessary to take smears for an adequate period of control, to assay urine for 17-ketosteroids and to follow

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these procedures after the administration of hormones. Further study along these lines is now in progress.

SUMMARY

1. Daily urethral smears were taken by 12 men, aged 18 to 32 years for periods of time ranging from 7 to 122 days; by 6 men, aged 44 to 58 years, for periods of time ranging from 3 to 50 days; and on 5 boys, 9 to 11 years old, every other day for one week.

2. The constituents of the 1056 urethral smears studied included cornified, superficial and intermediate cells derived from squamous epithelium and structures believed to be basal cells of the lower epithelial layers, which we have termed round cells. The superficial, intermediate and round cells exhibit both acidophilic and basophilic staining properties. Leucocytes, bacteria, occasionally spermatozoa and certain refractile bodies of unknown nature and origin were also present. A moderate amount of debris, believed to be of cellular origin was observed in all smears.

3. There was marked variation in the types of cells and in their staining properties present from person to person, and in the same individual from time to time.

4. The urethral smears of the subjects ranging in age from 18 to 32 years showed a somewhat higher degree of cornification and acidophilism than did those of the younger and older males. Moreover, the percentage of round cells and leucocytes was somewhat higher in this group.

5. No regularly cyclic variations in the urethral smear have been observed in any individual or in any age group.

6. The histology of the urethral smear and the factors on which it may be dependent have been discussed.

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EFFECT OF dl-α-TOCOPHEROL ACETATE ON PROGESTERONE METABOLISM

H. DE WATTEVILLE, M.D., R. BORTH, PH.D., AND M. GSELL, M.D.
Clinique universitaire de gynécologie et d'obstétrique, Geneva, Switzerland

THERE is a large amount of biological and clinical literature[1] on the possible interaction of progesterone and vitamin E, especially in pregnancy. This problem, however, seems still far from being solved (1, 2). To our knowledge, it has been dealt with by means of biochemical methods in only three reports. In these instances the authors used the determination of urinary sodium pregnanediol glucuronidate (NaPG) by Venning's method (3, 4). During the human menstrual cycle (5) and in imminent abortion (6), daily doses of vitamin E in the form of α-tocopherol acetate increased the NaPG output considerably. In normal pregnancy (7) the increase of NaPG output after *dl*-tocopherol acetate administration was not very great.

Huber's (8) new chromatographic method for routine determinations of urinary pregnanediol, with the modifications described in this paper, enabled us to investigate the problem under discussion on a larger experimental basis. The object of this study was to determine whether administration of *dl*-α-tocopherol acetate could affect the known part of progesterone metabolism. The transformation of progesterone into urinary pregnanediol was studied under conditions which excluded any influence of ovarian function during the menstrual cycle.

METHODS

Twelve postmenopausal women treated for uterine and breast carcinomas in our clinic were chosen for the experiments. In 11 of these patients, the uterus and ovaries were present; in 1 patient (case 19) both ovaries had been removed. All patients with carcinoma of the cervix had received radiation treatment. The usual urine examinations showed no signs of abnormal renal function. The patients received their usual diet. The essential data of the case histories are compiled in Table 1.

All 12 women received one intramuscular injection of 100 mg. of progesterone in oil.[2] There were no bad after-effects. Beginning with the day before the injection, pregnanediol was determined daily in twenty-four-hour samples of urine until its excretion ceased.

Following the disappearance of pregnanediol from the urine, 10 of the

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[1] For extensive review of literature, see Thesis, M. Gsell, Geneva, Switzerland, 1948.

[2] Lutocyclin (Ciba).

patients received one dose of 60 mg. of *dl*-α-tocopherol acetate in tablets[3] daily, for seven days. On the eighth day, the vitamin E level in the blood was determined.[4] On the same day, a second injection of 100 mg. of progesterone was given, and the excretion of urinary pregnanediol was determined daily, as after the first injection.

For control purposes, two patients received the second progesterone injection without preceding tocopherol treatment.

TABLE 1. RÉSUMÉ OF CASE HISTORIES

Case No. (1947)	Age Yrs.	Clinical diagnosis	Time in years since menopause	Urine analysis
19	64	Carcinoma of cervix, grade IV; bilateral ovariectomy	35	Cystitis
117	54	Carcinoma of cervix, grade II; cachexia, generalized metastases	4	—
175	63	Carcinoma of cervix, grade IV; cachexia, generalized metastases	9	—
241	53	Carcinoma of breast	1	Sugar (+)
361	52	Carcinoma of cervix, grade II	1	—
434	53	Carcinoma of cervix, grade III	4	Cystitis
532	40	Carcinoma of cervix, grade I	2	—
535	58	Carcinoma of cervix, grade III; ileus, diarrhea	6	—
542	81	Carcinoma of breast; slight cardiac insufficiency	25	Traces of albumin
554	78	Carcinoma of cervix, grade II	12	—
566	70	Relapse of carcinoma of breast, following surgical and radiation treatment	25	Slight cystitis
614	59	Carcinoma of cervix, grade II	10	Cystitis

At the instigation of the senior author (H. de W.) Huber (8) had developed a chromatographic method for the determination of urinary pregnanediol,[1] which we used and which we modified in the following ways: 1) The handling of the crude toluene extract was made easier, and

[3] Ephynal (Roche).

[4] The tocopherol determinations were carried out by the Laboratoire universitaire de recherches de diagnose clinique et de biologie expérimentale dans le domaine des vitamines, Lausanne, Switzerland. The method used is based on the work of Kofler (9, 10) and of Emmerie (11).

emulsions were avoided. 2) The rather rough evaporation to dryness of the toluene solution on a hot plate and under normal pressure was regarded as inadequate in steroid analysis. This process was easily omitted because toluene and benzene do not differ in their respective eluotropic properties (Trappe (12)). 3) For the elution of pregnanediol, a mixture of benzene and ethyl alcohol was found superior to the benzene-ether mixture used by Huber. The following technique, which gave the best results in recovery experiments was finally adopted:

Technique of Pregnanediol Determination.

One liter of urine from a twenty-four-hour specimen and 50 cc. of redistilled toluene are heated to boiling. One hundred cc. of concentrated hydrochloric acid is added slowly, and the mixture is refluxed for fifteen minutes, followed by rapid cooling under the tap. The clear urine layer is drawn off in a separatory funnel and re-extracted twice by gentle shaking with 25 cc. portions of toluene. The combined toluene extracts and emulsions are washed with two to four 25 cc. portions of 2 normal sodium hydroxide in 20 per cent sodium chloride solution, until the latter remains clear and the emulsion is broken down. Two final washings with 25 cc. of distilled water will remove the sodium hydroxide.

Working with pregnancy urines from the fortieth or sixtieth day of pregnancy onwards, when more than 6 to 8 mg. pregnanediol in a twenty-four-hour specimen can be expected, the hydrolysis and extraction should be done as follows:

100 cc. of urine, 50 cc. of toluene, and 10 cc. of concentrated hydrochloric acid are refluxed for fifteen minutes, followed by careful but thorough shaking and rapid cooling under the tap. The toluene layer and the emulsion are combined and washed with two 15 cc. portions of 2 normal sodium hydroxide in 20 per cent sodium chloride solution, and twice with 15 cc. of distilled water.

The alkali-free, clear toluene extract obtained from 100 or 1000 cc. of urine is heated on a hot plate until the last traces of water are dispelled. As the vapors of toluene and water form an azeotropic mixture, this will be achieved in a few minutes. The solution is allowed to cool down to 50–60° C., then 10 cc. of 0.5 molar sodium methoxide or hydroxide in methyl alcohol solution (prepared by dissolving 1.15 Gm. of sodium or 2.0 Gm. of sodium hydroxide in 100 cc. of dry methyl alcohol) is added, and the mixture is evaporated on the hot plate until the volume is reduced to 20 or 25 cc. The vapors can be sucked off conveniently through a glass funnel connected to a water pump. The toluene solution is allowed to cool and is filtered by suction through a Pyrex glass filter of medium porosity, and the precipitate is washed with three successive small amounts of hot toluene. The filtrate is concentrated on the hot plate to a volume of 4–6 cc. and allowed to cool.

A chromatographic column about 7–9 mm. in diameter and 10–15 mm. high is prepared with 200–300 mg. of aluminum oxide (Merck, activity I, according to Brockmann), by sedimentation in dry benzene. The toluene extract is allowed to percolate through the column (gentle pressure may be used but is rarely necessary), and the column is washed with 6 cc. benzene. Then the pregnanediol is eluted into a weighed round-bottom flask with 10 cc. of benzene-absolute ethyl alcohol (20:1). The residue obtained after thorough evaporation in vacuum on a water bath is decanted with 0.5–1.0 cc. of cold ether to remove colored impurities, and the remaining white crystalline pregnanediol is dried by evaporation in vacuum and in a desiccator, and finally weighed.

The column described will adsorb up to 6–15 mg. pregnanediol. If greater amounts are

expected or if the amount actually found is more than 6 mg., then a larger column should be used; or the toluene and benzene eluates should be concentrated and passed once more through a new column.

The melting points of the pregnanediol fractions isolated from 100 cc. samples of pregnancy urine are above 228° C. In most other cases they are above 220° C. They are not depressed after addition of authentic pregnanediol-3(α), 20(α) (melting point, 234–236° C.; Engel, Thorn and Lewis (13)). The diacetate is prepared in the usual way with acetanhydride and pyridine, and after one recrystallization from methyl alcohol it melts at 160° and 177–178° C., which is the double melting point of 3(α), 20(α)-diacetoxy-pregnane (13, 14).

With this method, up to thirty determinations a week can be made by two persons. The smallest amount to be determined exactly, varies from 0.2 to 0.8 mg., depending upon the amount of pigments present in various urines.

Recovery Experiments

In accordance with Huber's (8) findings, crystalline pregnanediol added to urines before hydrolysis, was recovered quantitatively (95–100 per cent).

TABLE 2. PREGNANEDIOL DETERMINATIONS IN TWO SAMPLES FROM THE SAME URINE SPECIMEN

No. of determination	Volume analyzed cc.	Volume in 24 hours cc./24 hrs.	Pregnanediol found mg.	Pregnanediol mg./24 hrs.
266	100	1500	0.7	10.5
	1000		6.9	10.4
298	100	1340	0.3	4.0
	1000		3.4	4.6
300	100	900	0.3	2.7
	800		2.0	2.3
411	100	2000	0.2	4.0
	1000		1.7	3.4
413	100	500	2.4	12.0
	400		11.0	13.8
417	100	1300	2.3	30.0
	100		2.3	30.0
432	100	1420	1.2	17.0
	100		1.0	14.0

In order to determine the loss which is to be expected from hydrolysis of the water-soluble conjugated compound, pure pregnanolone-free sodium pregnanediol glucuronidate was prepared (Sutherland and Marrian (15)) and used in recovery experiments. Invariably, 65–70 per cent of the theoretical amount of pregnanediol could be recovered. This is in accordance with the losses ascribed to hydrolysis of conjugated steroids by

TABLE 3. EXCRETION OF URINARY PREGNANEDIOL AFTER INJECTION OF 100 MG. PROGESTERONE, BEFORE AND AFTER TOCOPHEROL TREATMENT

Days after injection of progesterone	Case No. 19	117	175	241	361	434	532	535	542	554	566	614
	Excretion of pregnanediol (mg. per 24 hours) after injection of progesterone											
	Before tocopherol treatment											
0 (before inj.)	0	0	0	0	0	0	0	0	0	0	0	0
1	5.6	7.6	7.0	3.6	4.3	1.9	2.0	3.3	3.3	3.1	5.5	5.8
2	4.8	3.4	2.7	2.9	3.6	5.0	2.6	1.9	3.4	2.4	3.2	2.9
3	0	1.4	0.3	2.8	2.0	1.6	1.8	1.9	2.6	2.1	2.1	2.2
4	0	1.4	0	0	1.2	0	0.6	0.9	0.4	0.9	1.6	1.0
5	—	0	0	0	0	—	0.3	0	0	0	0.4	0
6	—	—	—	—	—	—	0	—	—	—	0	—
	After tocopherol treatment											
0 (before inj.)	—	0	—	—	—	—	—	—	—	—	*	*
1	5.2	6.3	8.5	4.5	5.9	4.9	4.8	4.8	5.0	4.8	4.3	5.3
2	4.9	5.0	7.7	4.2	4.5	3.9	2.1	3.6	3.1	3.2	2.6	3.3
3	3.0	4.8	1.4	3.9	0	3.4	1.9	1.6	2.2	2.4	2.8	2.2
4	1.7	3.4	0	1.2	0	2.7	2.0	1.1	1.8	2.0	1.2	1.1
5	0	0.4	—	0	0	0	0.7	0.4	1.2	0.9	0.6	0
6	—	0	—	—	—	—	0	0	0.7	0	0	—
7	—	—	—	—	—	—	—	—	0	—	—	—
Total before treatment	10.4	13.8	10.0	9.3	11.1	8.5	7.3	8.0	9.7	8.5	12.8	11.9
Total after treatment	14.8	19.9	17.6	13.8	10.4	14.9	11.5	11.5	14.0	13.3	11.5*	11.9*
Difference	+4.4	+6.1	+7.6	+4.5	−0.7	+6.4	+4.2	+3.5	+4.3	+4.8	−1.3	0

* The patients in cases 566 and 614 received no tocopherol treatment before the second injection.

different methods (16, 17, 18, 19, 20). Since, of all urinary steroids, direct recovery experiments of this kind have been carried out so far only with pregnanediol, and for other obvious reasons, we hold the view that no correction factor should be used to increase the values obtained with this method.

Finally, several pregnanediol determinations were made in two samples from the same urine specimen. The results listed in Table 2 agree reasonably with each other.

RESULTS

The results of the daily pregnanediol determinations are listed in Table 3. Figure 1 shows the total excretion of pregnanediol before and after tocopherol treatment, and in the two controls, after the first and after the second injection. In eight cases the tocopherol level in blood serum on the day of the second injection is given.

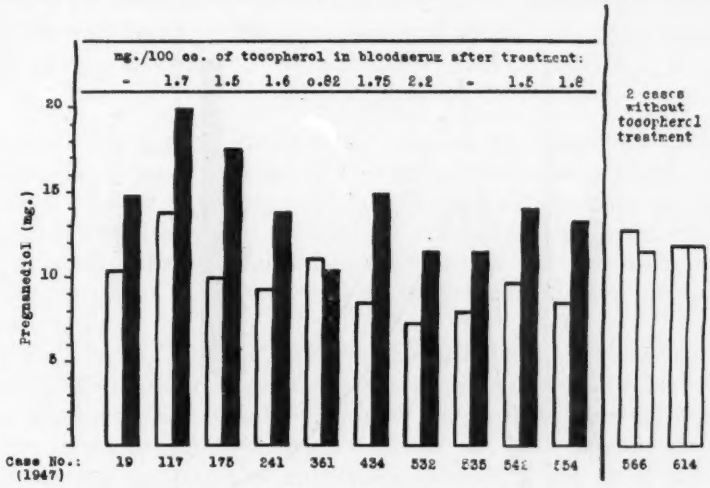
mg./100 cc. of tocopherol in bloodserum after treatment:
- 1.7 1.5 1.6 0.82 1.75 2.2 - 1.5 1.2
2 cases without tocopherol treatment
Pregnanediol (mg.)
20
15
10
5
Case No.: (1947)
19 117 175 241 361 434 532 635 541 554 566 614

FIG. 1. Total elimination of urinary pregnanediol after injection of 100 mg. progesterone. White columns—before tocopherol treatment. Black columns—after treatment with *dl*-α-tocopherol acetate. In eight cases the level of tocopherol in the blood after treatment is given (figures above columns).

In the 10 women treated with α-tocopherol acetate, of 100 mg. progesterone injected before treatment, 7.3–13.8 mg. (mean, 9.66 mg.) was recovered as urinary pregnanediol. After treatment, the amount recovered was 10.4–19.9 mg. (mean, 14.17 mg.). The mean increase was 4.51 mg. (*see* Fig. 2), and in each case the increase was always greater than one-third of the total excretion before treatment, with the one exception of case 361. Just as in the untreated controls (cases 566 and 614), there was in this case almost no difference between the total excretion after the first and after the second injection of progesterone.

Statistical Analysis

As usual, "Student's" *t*-test (Linder (21)) was used to decide whether the mean increase, 4.51 mg., in total pregnanediol excretion caused by

tocopherol treatment, was statistically significant. Since $t=6.446$ and $P<0.001$, it follows that the increase of 4.51 mg. is highly significant.

The mean durations of pregnanediol excretion before and after tocopherol treatment, 3.6 and 4.3 days respectively, do not differ significantly. But if case No. 361, the exceptional case discussed hereafter, is disregarded then the mean durations of excretion in the remaining nine cases are 3.56 and 4.56 days, respectively, and differ significantly ($t=4.23$ and $P<0.01$).

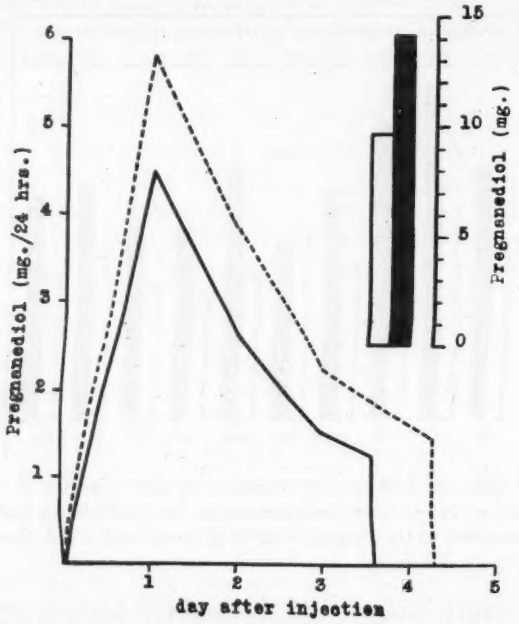

FIG. 2. Composite curves of daily excretion, and mean total excretion, of urinary pregnanediol in ten postmenopausal women, after injection of 100 mg. of progesterone. Full line and white column—before tocopherol treatment. Dotted line and black column—after treatment with *dl*-α-tocopherol acetate.

In order to integrate the twenty single excretion curves given numerically in Table 3 into two standard curves which would be typical for the excretion before and after treatment, the following procedure was used: When taking as abscissa the logarithm of the day after injection, and as ordinate not the daily excretion but the total excretion up to the corresponding day, nearly straight lines are obtained, and linear regression can be employed (21). Two over-all regression lines were calculated for the excretion before and after tocopherol treatment. Analysis of variance showed these two regressions to be linear ($F=1.031$ and 0.352, respectively,

and $P > 0.05$). By returning to the original variates, the two composite curves of Figure 2 were obtained. They demonstrate clearly the results presented in this paper.

Finally it follows from the analysis of variance (Fisher (22)) that the variance caused by tocopherol treatment is significantly greater than the variance due to the differences between the patients ($F = 7.15$ and $P < 0.01$) Therefore, the tocopherol effect presented in this paper could probably be demonstrated even if the two injections before and after tocopherol treatment were given not to the same but to different patients.

DISCUSSION

The result of our experiments is that *dl*-α-tocopherol acetate treatment increases the urinary pregnanediol output in postmenopausal women previously injected with progesterone. The exceptional case No. 361 is especially illuminating, because here the tocopherol treatment not only failed to increase the pregnanediol excretion but at the same time did not raise the tocopherol blood level beyond the normal range of 0.7–1.0 mg. per cent.

It is well known (Burrows (23)) that the endometrium is not necessary for the transformation of progesterone into pregnanediol. It seems worth noting, therefore, that at least in those of our patients who had received radiation treatment for carcinomas of the cervix, the endometrium must have been completely atrophic. Any follicle ripening, if not out of the question because of the patient's age, would also have been suppressed. The results obtained from the patients with breast carcinomas (cases 241, 542, 566) are wholly within the range of the other values.

In his interpretation of the effects of tocopherol treatment on pregnanediol excretion, Winkler (5, 6) assumed that increased pregnanediol output must *necessarily* originate from an increase in progesterone production. This assumption is incompatible with the results presented in this paper. Before and after tocopherol treatment, the body of each of our patients had at its disposal the same amount of injected progesterone. Ovarian progesterone production can be excluded on the evidence of the case histories. The stimulation of progesterone production by the adrenals (Burrows (23)), as tentatively conceived by Stähler and Hopp (24) among other assumptions, is also not likely to bring about the observed effects. The above reasoning is confirmed, to a certain extent, by the zero pregnanediol values before the injections. Therefore, the increase in pregnanediol elimination after tocopherol treatment has to be understood, at least in the present experiments, as evidence for a shift in the intermediary metabolism of progesterone. That is, its transformation into pregnanediol has been increased at the expense of the other pathways of progesterone

metabolism which are not known but which nevertheless account for the major part of progesterone inactivation in the liver, kidney, and spleen (25, 26, 27). Likewise, those chemical reactions of progesterone which must be supposed to accompany the biological action of this hormone in its target organ, the endometrium, are not known. We want to emphasize, therefore, that no rash conclusions should be drawn from our results with regard to the therapeutic value of α-tocopherol in imminent abortion, or with regard to the diagnostic value of urinary pregnanediol in women. Even if many metabolic factors admittedly influence the amount of pregnanediol excreted, it is evident from many years of clinical and biochemical experience that in most cases the pregnanediol level primarily depends upon, and is parallel with, the amount of progesterone present in the female body.

Investigation into these diagnostic and therapeutic problems is closely related to the biological significance ascribed to urinary pregnanediol. It cannot yet be decided whether this compound or unknown metabolites represent the amount of progesterone which has been biologically active, as distinct from the part merely inactivated; or if such a distinction can at all be drawn. Some evidence on this question, which is beyond the scope of this article, can be seen in the work on urinary pregnanediol output after progesterone and pregneninolone administration by mouth (28, 29, 30, 31).

Apart from this problem, the results of this paper can easily be interpreted in terms of the vitamin E theory of Hickman and Harris (1). Acting as an antioxidant, α-tocopherol hinders the destruction of progesterone by oxidation. If a larger amount of α-tocopherol is available, less progesterone is oxidized, and more of the reduced metabolite, pregnanediol, is eliminated in urine.

SUMMARY

1. In ten postmenopausal women, the excretion of urinary pregnanediol after injection of progesterone was increased by *dl*-α-tocopherol acetate treatment. The effect is statistically highly significant.

2. The result is interpreted as a shift in progesterone metabolism in favor of pregnanediol production, provoked by the antioxidative effect of α-tocopherol.

3. A method is described for the determination of as little as 0.2 to 0.8 mg. of pregnanediol in 100 or 1000 cc. of urine. Up to thirty determinations a week can be made by two persons.

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TURNER'S SYNDROME*

ROBERT B. GREENBLATT, M.D. AND
H. E. NIEBURGS, M.D.

From the Department of Endocrinology, University of Georgia School of Medicine, Augusta, Ga.

A SYNDROME of decreased stature, sexual infantilism, congenital webbing of the neck and cubitus valgus was described by Turner in 1938 (1). All of his seven patients were females and ranged in height from 50 to 56 inches and were between the ages of 15 and 23 years. All showed evidence of extreme prepuberal hypo-ovarianism for which Turner felt that the anterior pituitary gland was responsible. Roentgenograms showed delayed union of bones and evidence of demineralization in six of his cases. No hormone assays were obtained in his original series. Since Turner's first report, Varney, Kenyon and Koch (1942) reported on the association of short stature, retarded sexual development and high urinary gonadotropin titers in women (2). No congenital anomalies were noted in their series. In the same year, Albright, Smith and Fraser described eleven patients who had a syndrome of primary ovarian insufficiency and decreased stature (3). An excess of urinary gonadotropins was demonstrated in these cases. Furthermore, several of the patients exhibited features similar to those originally described by Turner.

Probably the earliest recorded case of webbed neck associated with sexual infantilism was that of a girl 15 years of age reported in 1902 by Funke (4). Up to 1938 a total of 40 such cases had been reported, chiefly from French and German sources. In 1941, Sharpey-Shafer (5) contributed another case, also in a female, with webbing of the neck, increased carrying angle of the elbow and sexual infantilism. Aside from one case reported by Flavell in 1943 (6), an example of Turner's syndrome occurring in a male subject has not been recorded in the literature. In 1944, Shereshevski described five cases with essentially the same characteristics as those of Turner's cases (7). Unfortunately, these were labelled as examples of pituitary dwarfism and hormone studies were not made. In 1945, Schneider and McCullagh collected a series of six cases of Turner's syndrome (8). They reported increased gonadotropin titers, differentiated their cases from pituitary dwarfism and suggested a similarity to ovarian dwarfism. More recently the contributions of del Castillo (9), Dorff (10), Lisser (11) and their associates have added to the knowledge of this syndrome in the female.

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* Aided by a grant from Ayerst, McKenna & Harrison, Ltd., New York, N. Y.

The purpose of this communication is to record another case of Turner's syndrome in a female with increased gonadotropin titer and more particularly, to report a second case of Turner's syndrome in the male. The finding of tubular hypoplasia of the testes in this patient may, by analogy, permit certain inferences as to the ovarian hypofunction in the female.

Case 1 is that of a colored girl 15 years of age with decreased stature (Fig. 1). Her height was 47 inches, span 48 inches and weight 54 pounds, which correspond to the

FIG. 1. Case 1. Note decreased stature, absence of secondary sex characteristics, webbing of the neck and moderate degree of cubitus valgus.

upper normal limits for a child 6½ years of age. The span was increased in relation to the height by about two inches. Her mental condition seemed fairly normal. She showed characteristic features of Turner's syndrome, i.e. webbing of the neck, low hair line, sexual infantilism and slight increase in carrying angle. Clinical examination revealed no further abnormalities. There was no coarctation of the aorta. The vaginal smears showed basal cells characteristic of an atrophic vaginal mucosa (Fig. 2). Blood studies

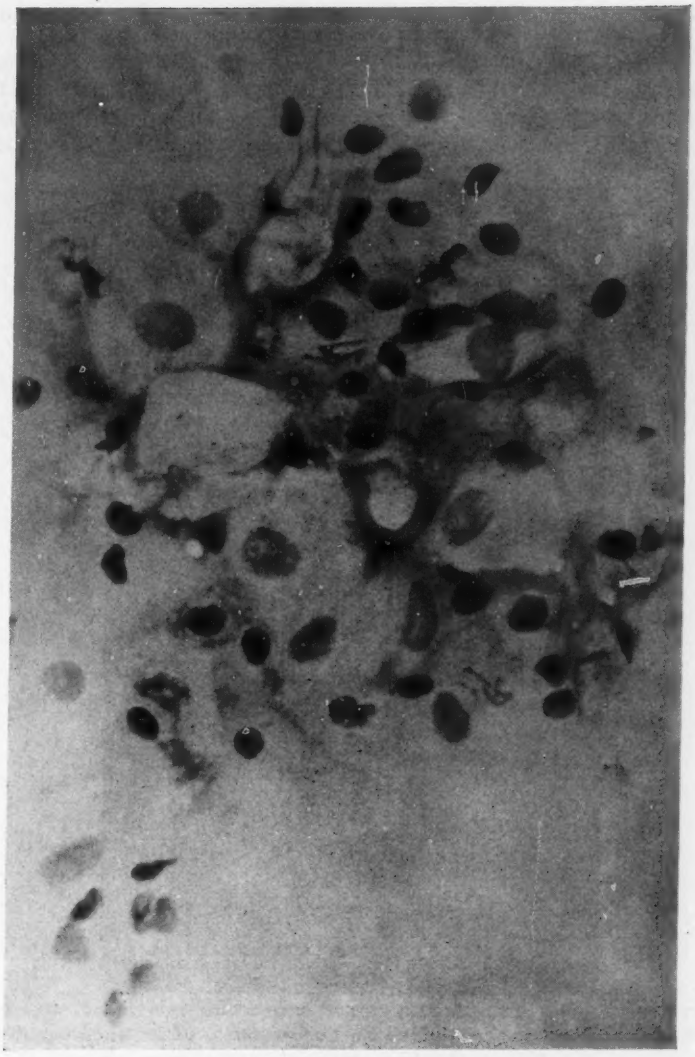

FIG. 2. Case 1. Vaginal smear, before treatment. Note basal or castrate cells. ×380.

revealed inorganic phosphorus values of 5.21 mg. per cent, and calcium, 11.6 mg. per cent. The insulin tolerance test was within the range of normal. The basal metabolic rate was plus 1 per cent. The blood count was normal. Urinary 17-ketosteroid assay by the method of Holtorff and Koch (12) revealed 0.5 mg. per 24-hour specimen, whereas

the normal range for females in this laboratory is 5 to 18 mg. per 24 hours, with an average of 9 mg. per 24 hours. The blood gonadotropin assay revealed a markedly increased amount of follicle-stimulating hormone (FSH). Undiluted serum injected in a dose of 4 cc. over four days to each of four 21-day-old female mice (Fluhmann's method (13)) showed marked follicle stimulation with a fivefold increase above normal in ovarian and uterine weight. The report of x-ray studies was as follows: No definite evidence of mal-

FIG. 3. Case 1. Roentgenogram of left hand showing retardation of osseous development. Epiphyses of radius, ulna and phalanges remain open.

formation of the skull except for its brachiocephalic type. Teeth appear normal for age. Anteroposterior and lateral views of the cervical spine show slight elongation of the transverse processes of the seventh cervical vertebra with slight upper dorsal scoliosis. Anteroposterior views of both wrists show the left wrist slightly flexed toward the ulnar side, which finding might be positional. The pisiform bone appears normal. Osseous development is delayed by two or three years (Fig. 3). The sella turcica appears to be slightly large but otherwise normal. Both shoulders appear to be high, which findings may be due to position or upward displacement of the scapula.

Oral estrogen therapy was instituted over one year ago. She is being maintained on 0.625 mg. of estrone sulfate[1] daily for twenty-one days in each month. During this period there has been an increase in height of 1½ inches, to 48½ inches; an increase in weight of about 10½ pounds, to 64½ pounds; and an increase in breast growth along with the appearance of pubic hair and occasional menstrual periods.

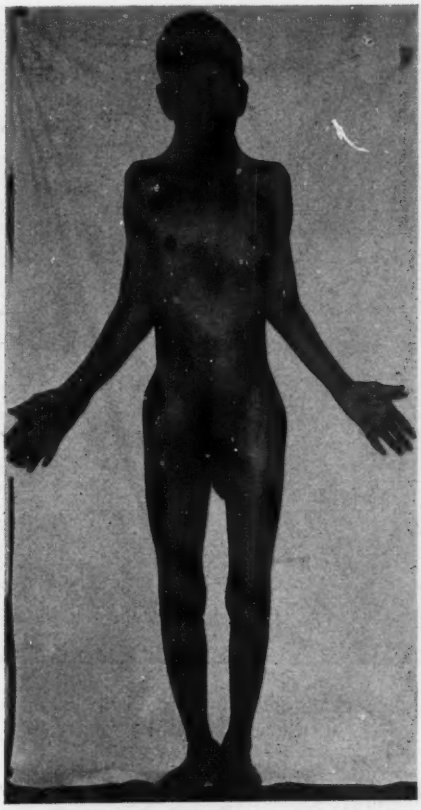

FIG. 4. Case 2. Note dwarfism associated with poor muscular development, webbing of the neck, marked increase in carrying angle and, paradoxically, enlarged phallus.

Case II is that of a colored male, 30 years of age, with dwarfism (Fig. 4). His height was 57 inches and his weight 76 pounds, which correspond to the upper limits for an 11-year-old child. He showed webbing of the neck, markedly increased carrying angle of the arms (cubitus valgus) (Fig. 5), slight talipes varus, poor muscular development

[1] The estrone sulfate used in this study was supplied to us in the form of "Premarin" by Ayerst, McKenna & Harrison, Ltd.

(particularly of the upper extremities), moderately poor beard growth, small testes with atrophy of the left testicle and, paradoxically, a very large penis. The prostate seemed to be small on palpation. There was no coarctation of the aorta. The patient's chief complaint was of muscular weakness and inability to work. X-ray plates of the wrists and hands showed decreased size of the bones of the forearm; all centers of ossification were

FIG. 5. Case 2. Roentgenogram of right elbow showing increased carrying angle due to an outward bending of the humerus.

present and the epiphyses were united. X-ray examination of the skull revealed no abnormality. The basal metabolic rate was plus 10 per cent. The seminal fluid volume was one-half cubic centimeter and azoospermia was present. Biopsy of the right testis revealed hypoplasia of the seminiferous tubules, azoospermia and a relative increase in the number of interstitial cells (Figs. 6, 7, 8). Two urinary 17-ketosteroid determinations revealed 9 mg. and 11.9 mg. per 24-hour specimen. The normal range for males in this laboratory is 8 to 28 mg. per 24-hour specimen. Serum gonadotropin assays showed no increased values.

COMMENT

Case 1 seems to confirm the recent evidence (3, 5, 14) that this syndrome is accompanied by primary ovarian deficiency. Whether substitu-

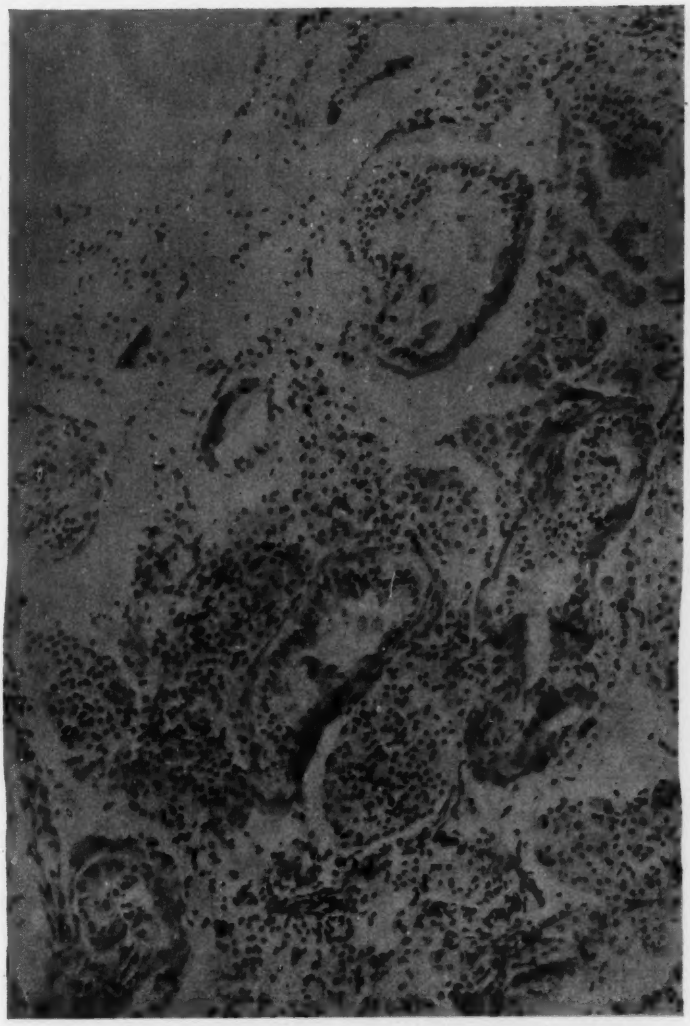

FIG. 6. Case 2. Biopsy specimen of right testis, showing tubular hypoplasia with seemingly increased numbers of interstitial cells. ×90.

tional estrogen therapy will result in a marked increase in growth must await further observation. The cases originally described by Turner did not respond to pituitary growth hormone. However, following the administration of anterior pituitary gonadotropic hormone, there was definite improvement in the patients so treated.

FIG. 7. Case 2. One tubule, from biopsy specimen of right testis. ×380.

In the second case it may be conjectured that the testicular tubular hypoplasia is of primary nature since the interstitial cells could not have been maintained if there was pituitary failure. The failure to obtain positive assays does not obviate such a conclusion, since negative blood gonadotropin findings are not only obtained in cases of hypopituitarism but also in normal individuals. The 17-ketosteroid excretion is within the

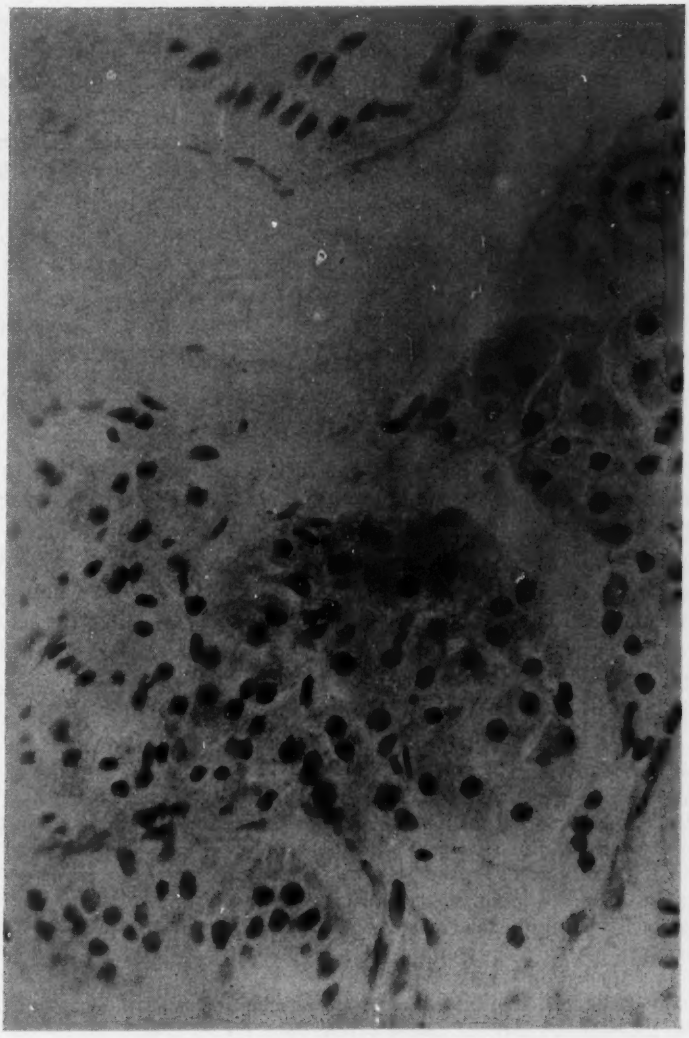

Fig. 8. Case 2. Interstitial cells, from biopsy specimen of right testis. ×380.

limits of normal and not high enough to explain the increased size of the phallus. It was thought that the patient's adrenals might be responsible for increased androgen production although such an assumption does not fit in with the relatively low 17-ketosteroid excretion, the muscular weakness and the scanty body and facial hair growth. Perirenal insufflation re-

vealed that both adrenals were of normal size and shape. It was not possible to obtain a definite statement as to the patient's libido, but it appears that his requirements are rather minimal for he sought sexual congress but once every three to six months.

Flavell's case was that of a white male 21 years of age with marked webbing of the neck, increased carrying angle of the elbow, some kyphoscoliosis and third degree claw feet. Along with scanty beard and no axillary hair, the patient had a feminine distribution of pubic hair. Both testes were small and hypoplastic. The penis was of normal size and it was claimed that erections and emissions were normal.

It might be conjectured that in our case the enlarged phallus is one of the congenital anomalies present and is as difficult to understand as the webbing of the neck and the cubitus valgus.

SUMMARY

Another case of Turner's syndrome in the female is recorded in which increased gonadotropin titers were found. Signs of marked hypo-ovarianism were present.

A case of Turner's syndrome in the male is presented. Evidence of hypogonadism was substantiated by biopsy of the testis, which showed hypoplasia and azoospermia with seemingly increased Leydig cells. This case of Turner's syndrome in the male is the second to be reported in the literature.

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The Association Postgraduate Course In Endocrinology

THE Postgraduate Committee of THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS, under authority of its Council, announces a course of lectures and demonstrations in CLINICAL ENDOCRINOLOGY to be held in OKLAHOMA CITY at the SKIRVIN HOTEL, FEBRUARY 21 to 26, inclusive, 1949.

The faculty will consist of the most prominent investigators and clinical endocrinologists in the various branches of the medical sciences in the United States and Canada.

It is the intent of the Committee that this course be a practical one of interest and value to both the GENERAL PRACTITIONER AND THE SPECIALIST.

A fee of $100 will be charged for the entire course and the attendance will be limited to 100. Registration will be in the order of checks received. Should there be an insufficient number of applicants to warrant the course, the registration fee will be immediately refunded in full.

Please make your application on your letterhead and forward, together with your check payable to THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS, to DR. HENRY H. TURNER, CHAIRMAN OF THE POSTGRADUATE COMMITTEE, 1200 NORTH WALKER STREET, OKLAHOMA CITY 3, OKLAHOMA.

Due to other meetings being held in Oklahoma City at the time of this assembly, satisfactory hotel accommodations will be difficult to procure on short notice; therefore, it is suggested that all applicants MAKE THEIR RESERVATIONS EARLY.

SOME OF THE BETTER DOWNTOWN HOTELS IN OKLAHOMA CITY ARE: Biltmore, Black, Huckins, Skirvin, Skirvin Tower, Wells-Roberts.

The 1949 Meeting of the Association for the Study of Internal Secretions

The Thirty-First Annual Meeting of the Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3 and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3, at 7 p.m., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send the title and four copies of an abstract of not more than 200 words, to Dr. J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete, with results and conclusions, in order that they may be of value for reference and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be filed on special forms with the Secretary of the Association, not later than March 15, 1949, according to specifications given in the section on Awards.

The Awards of the Association for 1949

Nominations for Awards

Three Awards for meritorious work in endocrinology will be given at the next annual meeting of the Association. A special committee of five members of the Association chooses the recipients of these Awards, subject to ratification by the Council, and each member of the Association has the privilege of making one nomination for each Award.

Nominations for the Awards should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City 3, Oklahoma. All nominations, accompanied by a statement of the importance of the nominee's contributions to endocrinology and a bibliography of his most important papers with reprints if possible, should be sent to Dr. Turner's office not later than March 15, 1949.

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb and Sons Award of $1,000.00 was established in 1939. It was given in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. Edward A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori and in 1948 to Dr. Fuller Albright. No award was made in 1943. No age or special limitation is stipulated by the donor of the Award.

THE CIBA AWARD

The Ciba Award, established in 1942, is given in recognition of the meritorious accomplishment of an investigator, not over 35 years of age, in the field of clinical or preclinical endocrinology. In 1944 the Award was given to Dr. E. B. Astwood; in 1945 to Dr. Jane Anne Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li and in 1948 to Dr. Carl G. Heller. The Award is for $1,200.00. If within two years of the date of the Award, the recipient chooses to use it to aid in working in a laboratory other than the one in which he normally is located, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The first Ayerst, McKenna & Harrison Fellowship was given to Dr. Samuel Dvoskin in 1947 and in 1948 it was given to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2500.00. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals, submit a program of proposed study, indicate one or more institutions where the proposed program shall be carried out and submit statements of approval from the investigators with whom he proposes to conduct his research.

Notes on the Thirtieth Annual Meeting of the Association for the Study of Internal Secretions

LIST OF OFFICERS, COUNCIL AND COMMITTEE MEMBERS

The Thirtieth Annual Meeting was held in the Palmer House, Chicago, Illinois, June 18 and 19, 1948.

Forty-seven papers were presented and forty-four papers were read by title. Total registration was 458, equally divided between members and non-members. Two hundred and twenty-two were present at the dinner at which the presidential address was given by Dr. C. N. H. Long.

Actions of general interest taken by the Council are as follows:

(1) It was the decision of the Council to hold a Postgraduate Assembly in Oklahoma City, February 21–26, 1949, similar to the one which was so well received in Los Angeles in 1948.

(2) The Council approved the preparation and publication of an index for Volumes 1 to 40 of ENDOCRINOLOGY. This will be published by Mr. Charles C Thomas. The index for Volumes 1 to 26 will appear first; that for Volumes 27 to 40 will appear as soon as possible thereafter.

(3) It was the Council's decision to publish the Transactions of the American Goiter Association.

(4) The Council accepted with much regret the resignation of Dr. Earl T. Engle, Chairman of the Publications Committee, and appointed Dr. Warren O. Nelson to this office. Appreciation was expressed to Doctor Engle for his work as chairman of this committee.

(5) The Committee on Registry of Endocrine Pathology was reappointed to negotiate with the Scientific Director of the American Registry of Pathology regarding the formation of an Endocrine Registry at the Army Institute of Pathology. A sum not to exceed $750.00 was appropriated for necessary expense for one year.

(6) It was voted to appropriate $250.00 for 1949 to the National Society for Medical Research.

(7) Owing to increasing cost of labor, paper and all printing materials, and the enlargement of the Journals, the dues were increased to $11.00 per year which includes subscription to either of the Journals, with a combination offer of $16.00 for both Journals.

(8) The Council voted that the Thirty-first Annual Meeting be held June 3 and 4, 1949, in Atlantic City, New Jersey.

(9) A list of the 1948–49 Officers, Council and Committee members follows:

Officers

President	John S. L. Browne
President-Elect	Edward A. Doisy
Vice President	James H. Means
Secretary-Treasurer	Henry H. Turner

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Carl R. Moore
Gregory Pincus
C. N. H. Long
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Roy G. Hoskins
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W. O. Nelson, Chairman
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Managing Editors

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Willard O. Thompson—Journal of Clinical Endocrinology

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Committee on Nomenclature

Elmer Sevringhaus, Chairman
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Thomas McGavack

Consultant Committee to Food and Drug Administration

Allan T. Kenyon, Chairman
George Sayers
J. P. Pratt

Award of the American Goiter Association

THE VAN METER PRIZE

The American Goiter Association again offers the Van Meter Prize Award of three hundred dollars and two Honorable Mentions for the best essays submitted concerning original work on problems related to the thyroid gland. The Award will be made at the annual meeting of the Association which will be held in Madison, Wisconsin, May 26, 27 and 28, 1949, providing essays of sufficient merit are presented in competition.

The competing essays may cover either clinical or research investigations, should not exceed three thousand words in length, and must be presented in English. A typewritten double spaced copy should be sent to the Corresponding Secretary, Dr. T. C. Davison, 207 Doctors Building, Atlanta 3, Georgia not later than March 15, 1949. The Committee, who will review the manuscripts, is composed of men well qualified to judge the merits of the competing essays.

A place will be reserved on the program of the annual meeting for presentation of the Prize Award essay by the author, if it is possible for him to attend. The essay will be published in the annual Transactions of the Association. This will not prevent its further publication, however, in any journal selected by the author.

Abstracts of
CURRENT ENDOCRINE LITERATURE

Editor: ROY HERTZ. *Collaborators:* A. R. ABARBANEL, F. N. ANDREWS, B. L. BAKER, F. A. DE LA BALZE, ISRAEL BRAM, R. A. CLEGHORN, RUCKER CLEVELAND, C. D. DAVIS, ANNA FORBES, M. B. GORDON, H. S. GUTTERMAN, M. M. HOFFMAN, R. G. HOSKINS, C. D. KOCHAKIAN, H. S. KUPPERMAN, H. L. MASON, JANET W. MCARTHUR, THOMAS H. MCGAVACK, A. E. MEYER, K. E. PASCHKIS, A. B. PINTO, J. R. REFORZOMEMBRIVES, E. C. REIFENSTEIN, JR., G. G. RUDOLPH, L. T. SAMUELS.

ADRENALS

RAVREBY, LOUIS, and SAWYER, W. H.: Addison's disease complicated by chronic glomerulonephritis, *New England J. Med.* **239**: 110–113, 1948.

A male patient 55 years of age presented pigmentation and superimposed dermatitis. The simultaneously present renal disease complicated the diagnostic problem. Loss of weight and hypoglycemia were not present, the blood chloride was at a low normal level and the blood pressure showed a moderate hypertension which was sustained until the patient went into an Addisonian crisis. At this stage, sodium chloride, glucose and cortical hormone were without effect. The autopsy findings confirmed the diagnosis of nephritis and Addison's disease.—*A.E.M.*

GENERAL

BENSON, R. C.: The arrest of abnormal uterine bleeding with pitressin tannate in oil, *Am. J. Obst. & Gynec.* **55**: 286–292, 1948.

The author treated 100 consecutive gynecologic patients, excluding only those with malignancy, suffering from excessive uterine bleeding, with pitressin tannate in oil, 10 pressor units, intramuscularly. Thirty-one ceased to bleed in 24 hours and 17 more stopped in 48 hours. Thirty-five others responded in 48 hours and only 16 failed to notice any decrease in the bleeding within 48 hours. Patients with bleeding complications of pregnancy did not respond as well as those who had excessive uterine bleeding on the basis of functional or constitutional conditions. No toxic symptoms were noted, as care was taken to eliminate patients with severe nephritis, hypertension or cardiac disease. Endometrial biopsy before and after treatment showed no change in endometrial pattern.—*C.D.D.*

BOWES, K.; RITERBAND, S. H., and ANDREWS, J. E.: Demonstration by infrared photography of the superficial veins in the pregnant and nonpregnant woman, *J. Obst. & Gynaec. Brit. Emp.* **55**: 285–292, 1948.

Superficial veins of the breast, abdomen, legs and vulva were studied. No changes were observed during the menstrual cycle, though after the menopause the mammary veins become less marked. During pregnancy veins enlarge, the enlargement of those in the breast occurring first, within three to four weeks, and being the most marked. There

is also an increase in the number of anastomoses. These changes are so characteristic in the breast veins that they might be utilized as a rapid, simple, and cheap test of pregnancy.—*R.A.C.*

IMBRIANO, A. E.: Testosterone propionate in chronic nephritis, *Semana méd.* **55:** 750–758, 1948.

Six patients with chronic nephritis were treated with daily injections of testosterone propionate, given in an average dose of 25 mg. daily for 12 days. The results were evaluated by urine analysis and by the tubular reabsorption test with antidiuretic hormone. The tubular reabsorption test, markedly abnormal before treatment, showed gradual improvement finally reaching normal values. The diuresis increased to a variable degree. The urinary protein disappeared in two patients and was diminished to a trace in the others. Blood cells and casts disappeared in all cases.—*A.E.M.*

KUNDE, M. M.: The role of hormones in the treatment of obesity, *Ann. Int. Med.* **28:** 971–988, 1948.

Fifty obese female patients, varying in age between 34 months and 55 years, were studied with regard to basal metabolism, blood cholesterol, blood calcium, blood phosphorus, glucose tolerance test, roentgenogram of sella turcica, and blood pressure. These tests were repeated whenever possible at the end of the period of weight loss. No patients with diabetes or marked hypothyroidism were included. In all these patients there had been a previous diagnosis of endocrine obesity, and many exhibited body configurations corresponding to types of so-called endocrine obesity. The emphasis in treatment was not reduction in caloric intake, but reeducation of the appetite. No calculated caloric diet was suggested. A list of food substances chosen to constitute a high-protein, low-fat, low-carbohydrate diet was prescribed. Supplementary vitamins and minerals were given. The patients were cautioned concerning additional salt. All patients lost weight on the above regimen. No endocrine product was administered routinely. The author cautions against the use of thyroid substance except for the correction of a specific hypothyroidism. The weight loss was in several instances well over 100 pounds. Glucose tolerance determinations indicated an initial reduced glucose utilization in most of the patients. The majority showed a more normal glucose tolerance curve after 4 to 6 months of the dietary regimen, during which time a loss of 40 to 60 pounds had occurred. Some patients had an appreciable reduction in both systolic and diastolic blood pressure after 30 to 60 pounds had been lost.—*C.D.D.*

LAJOS, L., and SZONTAGH, F.: Evolution and growth of tadpoles by feeding vernix caseosa, progesterone, and folliculin, *J. Obst. & Gynaec. Brit. Emp.* **55:** 281–285, 1948.

Previous studies by the authors showed that vernix caseosa had a powerful estrogenic effect but no gonadotropic action. In the present study it is shown that the addition of ten per cent vernix to the diet of tadpoles was followed by an increase in growth and markedly accelerated metamorphosis. Estrogenic hormones do not produce these effects but the literature indicates that combined thymus and thyroid feeding does produce them.—*R.A.C.*

MacKenzie, L. L.; Welchler, B. B.; DuBois, J. C., and Neustaeder, T.: The use of the vaginal smear in a gynecological service, *Am. J. Obst. & Gynec.* **55**: 821–830, 1948.

In 76 menopausal patients, the vaginal smear agreed with the clinical diagnosis in 82 per cent of those with a natural menopause and in 81 per cent of those with a surgical menopause. In the latter group an average of 47 per cent of the smears showed atrophy, whereas in the former only 37 per cent showed this condition. Regardless of the etiology of the menopause, 35 per cent of the smears showed atrophy within 18 months, and 60 per cent showed atrophy after this time. The authors found, using Papanicolaou's criteria, an 84 per cent agreement between the known phase of the menstrual cycle and a single vaginal smear. The vaginal smear correlated with the endometrial biopsy 89 per cent of the time. The authors feel that the vaginal smear helps to establish the prognosis in secondary amenorrhea, as they had very little success in treatment of the patients with definite atrophic changes. The authors also report their experience with the use of the vaginal smear in gynecologic malignancy.—*C.D.D.*

Nylen, G. H., and Lavender, M.: Studies on the circulation with the aid of tagged erythrocytes in a case of orthostatic hypotension (asympathicotonic hypotension), *Ann. Int. Med.* **28**: 723–746, 1948.

The authors studied a 69-year-old man who had orthostatic hypotension. They found that adrenalin caused a considerable acceleration of pulse and rise in blood pressure but that atropine was without effect. With the aid of labelled corpuscles (technique published elsewhere) the authors found that in this condition the circulation in the erect position is much slower than in normal subjects. They propose that typical cases of postural hypotension be called asympathicotonic hypotension, as the fundamental etiologic factor appears to be the absence of reflex regulations of blood pressure and pulse rate.—*C.D.D.*

Schmitz, H. E., and Towne, Janet E.: The treatment of pelvic endometriosis, *Am. J. Obst. & Gynec.* **55**: 582–590, 1948.

The authors report on surgical, irradiation and hormonal treatment of 130 patients, averaging 32.6 years in age, with pelvic endometriosis. Ten required removal of both ovaries. Forty-seven had one or both ovaries preserved and 11 later conceived giving birth to 13 infants. This constitutes a corrected incidence of pregnancy of 27.5 per cent in those treated by conservative surgery. Roentgen therapy was used in a dosage sufficient to produce permanent menolysis in 17 patients; and 12 were treated with smaller doses, causing amenorrhea for 3 to 8 months. In this latter group, 2 conceived and delivered 3 infants. Roentgen therapy proved satisfactory for secondary therapy when conservative surgery had failed. Fifteen patients were treated with androgens; 6 had complete relief and 8 had partial relief. These patients had minimal pathology and the diagnosis was made clinically. The authors believe that male hormone therapy is of value in young women with mild cases of the disease. Twenty-five patients received no treatment and 19 patients were not reported upon.—*C.D.D.*